Host-symbiont specificity:

Exploring the dynamics of leaf-cutter ant-*Pseudonocardia* associations

by

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DISSERTATION ABSTRACT

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Symbiosis, the living together of unlike organisms, is critically important to the evolution of life on Earth. Virtually all animals host beneficial microbial symbionts, some of which are critical for normal host development and survival. Leaf-cutter ants farm a fungal cultivar as their primary nutrient source. The ants also carry a specialized Actinobacterial exosymbiont, in the genus *Pseudonocardia*, which produces antifungal compounds that inhibit *Escovopsis*, a parasite specialized on the ants' garden. In this dissertation, I use the leaf-cutter ant-*Pseudonocardia* mutualism as a model for host-beneficial microbe symbiosis, exploring across three scales: from genus to individual to genetic interactions. In chapter two, I employ 11 culturing techniques to explore whether *Atta* leaf-cutter ants, which lack visible exosymbionts, associate with *Pseudonocardia*. I isolated *Pseudonocardia* from all colonies attempted using at least one method, with bead-beating of workers being the most efficient. An Elongation Factor-Tu phylogeny placed three strains into ant-associated clades, with the majority falling into two novel clades. Additional work will be needed to localize and further characterize these strains. In chapter three, I used subcolony experiments to isolate the components and timing needed for proper exosymbiont acquisition in *Acromyrmex* ants. Major workers carrying visible *Pseudonocardia* need to interact with newly eclosed ants in a narrow window of a few hours after eclosion in order for successful transmission to occur. These constraints on colonization are

apparently mechanisms to ensure specificity. Specificity-producing mechanisms seem likely to occur in many symbioses to prevent host exploitation. In chapter four, I examined gene expression in symbiotic and aposymbiotic ants ten days after eclosion. I found differences in oxidation-reduction processes, reflecting established changes in aposymbiotic ant respiration rates. I also found upregulation in chitin metabolism, likely linked to host-associated molecular patterns. Finally, I found upregulation in aposymbiotic ants' NF-Kappa B pathway, perhaps corresponding to lower numbers of all non-ant reads including the ants' fungal cultivar food source, associated garden bacteria, and known bacteria symbionts across the cost-benefit spectrum, with *Pseudonocardia* the most prevalent. This work highlights the importance of specificity in ant-*Pseudonocardia* interactions and the likely importance of specificity as a first principle in symbiosis.

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TABLE OF CONTENTS

CHAPTER 1. BENEFICIAL MICROBE-HOST ASSOCIATIONS

I. Conceptual framework for studying symbiosis in an evolutionary context

In 1879 Heinrich Anton de Bary first defined the term symbiosis as "unlike organisms living together". This broad definition encompasses diverse interactions between hosts and their symbionts across the cost-benefit spectrum. Symbiotic relationships range from those in which both partners benefit (mutualism), to those in which a single partner benefits while the other appears to neither be harmed nor helped (commensalism), to those in which the symbiont benefits at the detriment of its host (parasitism). Over the course of an organism's lifespan these interactions can be obligate (necessary for the survival of one or both organisms) or facultative (occasionally arising but not required for survival). Symbiotic associations have played vital roles in the evolution of life on Earth. For example, endosymbiosis enabled the evolution of the eukaryotic cell from prokaryotic progenitors (Margulis 1981), leading to the development of both mitochondria in the earliest eukaryotes as well as chloroplasts in early plants (Dyall 2004). Symbiotic associations are common in nature and include a broad range of taxa from widespread infections of a diverse array of insects by *Wolbachia* to highly specific *Euprymna-Vibrio* colonization (reviewed in Werren *et al*. 2008; Nyholm & Mcfall-Ngai 2004). These associations continue to play a role in diverse evolving communities today. However, aside from a few symbiotic model systems, little is known about how hosts identify, establish and sustain their symbionts (reviewed in Bright & Bulgheresi 2010; Medina & Sachs 2010).

Mechanisms to ensure transmission of the symbiont to new hosts are critical to the establishment and maintenance of symbioses. In vertical transmission, hosts pass their symbionts directly to their offspring, evolutionarily linking the two. In addition, this transmission mechanisms is

theorized to align the interests of hosts and their symbionts by limiting partner choice and promoting partner fidelity (Bull & Rice 1991). The shared inheritance produced by vertical transmission also provides the partners greater opportunity to coevolve, or reciprocally adapt to one another (Thompson 1994). For example, many insects carry endosymbionts that share metabolic pathways with their hosts—relying on one another for essential anabolic and catabolic pathways (Moran 2006; *c.f.* MacDonald *et al*. 2011). This interdependence between host and symbionts may lead to reduced symbiont genome size since hosts can provide many basic functions for the symbiont (Moran 2006). Vertically transmitted symbiotic associations may be obligate, as in the example above, or facultative to the host such as the pea aphid-*Serratia* symbiosis in which the hosts benefit from additional heat tolerance (Burke *et al*. 2009).

Not all symbioses are transmitted vertically. The proximity and the resulting interactions between species essential for symbionts to have the opportunity to adapt to one another over evolutionary time scales can also be facilitated by horizontal transmission (Genkai-Kato & Yamamura 1999). In this transmission mechanism, hosts acquire their symbionts from the environment, typically early in the hosts' lifecycle. Horizontally transmitted symbionts include the *Euprymna-Vibrio fischeri* symbiosis, in which the newly hatched squid recruit their luminescent bacterial symbionts from the sea water around them (reviewed in Nyholm & Mcfall-Ngai 2004).

Symbioses vary in their degree of coevolution. The many endosymbionts with reduced genomes fall at the tightest end of the coevolutionary spectrum (Moran 2006). In contrast, horizontally transmitted, shared symbionts such as arbuscular mycorrhizal fungi and land plants are often loosely coevolved (reviewed in Brundrett 2002). Furthermore, hosts provide exosymbionts with

partially sheltered niche, however, these species frequently interact with, and can feel selective pressures from, the environment regardless of transmission route.

Evolutionary modeling anticipates hosts will derive the greatest benefit from symbiotic associations when their symbionts are not competing amongst themselves for resources (Frank 1996). By extension, this theory suggests evolution will favor limited microbial diversity within these associations. While, there are examples suggesting of nonclonal symbiont transmission, including diverse populations of medical leech crop *Aeromonas* symbionts (Silver *et al*. 2007), experimental evidence supports the prediction of limited diversity in several systems. For example, individual *Steinernema* nematodes can by colonized by one or two *Xenorhabdus* bacterial cells (Martens *et al*. 2003). Similarly, Wollenberg and Ruby (2009) demonstrated that the crypts of *Euprymna* squid are colonized by at most two individual *Vibrio fischeri* bacteria. Andersen used pyrosequencing to show that *Acromyrmex* leaf-cutter ants host *Pseudonocardia* from either one of two clades (2011), providing support for limited transmission in these ants.

Many studies have focused on game theory and models such as the Prisoner's Dilemma to model stability within symbiosis. Researchers theorized that evolution should favor cheating by one partner or the other, so cooperation should be rare in nature. However, recent work has revealed that many of these systems are far more complex, involving multiple interacting players (Little & Currie 2009; *c.f*. Pellmyr & Huth 1994).

Acquisition and maintenance. For colonization with a specific symbiont to occur, hosts must first be able to recognize their beneficial symbionts. Aspects of host colonization mechanisms have been worked out in a handful of symbioses, including *Euprymna-Vibrio* and *Steinernema-*

Xenorhabdus. Most symbionts are associated with specific host tissues (reviewed in Chaston & Goodrich-Blair 2010). This localization specificity appears to be mediated through physical interactions between hosts and symbionts, most prominently surface sugars and lectin proteins (*c.f.* Hooper & Gordon 2001). This type of interaction has been documented in *Steinernema-Xenorhabdus* (Martens & Goodrich-Blair 2005) and *Euprymna-Vibrio* (Nyholm 2000) and are theorized to occur in many other systems, including giant hydrothermal vent tube worms (*Riftia pachyptila*), Yeti crabs (*Kiwa hirsuta*) and earthworms (*Eisenia fetida*) (reviewed in Chaston & Goodrich-Blair 2010). Chitin, predominant in cell surfaces across many taxa, often plays a role in host-symbiont signaling. These sugar or lectin interactions can occur with either host- or microbe-associated molecular patterns (HAMPs or MAMPs) (reviewed in Mackey & McFall 2006). Hosts are thought to use two strategies to recognize their symbionts—1) a generalized recognition step, followed by a winnowing step to a single symbiont or 2) a single specific initial recognition step followed by colonization.

Once hosts have acquired their symbionts, they must actively maintain them. As in the colonization phase discussed above, this requires immune system fine-tuning to ensure symbionts are not attacked by their host (McFall-Ngai 2007). However, hosts may employ general antimicrobial molecules and pathways to manage their symbionts (Chun *et al*. 2008; Login *et al*. 2011). Hosts can also manipulate their own metabolic pathways to provision their symbionts with nutrients and/or receive support from their symbionts. This process is often tightly interwoven with host development (reviewed in McFall-Ngai 2002; Altura *et al*. 2011).

Stability. Individual hosts often must maintain their symbionts over their lifespan, however, for mutualisms to evolve populations of hosts must maintain symbionts over evolutionary time.

Hosts and symbionts often trade services in several broad categories including defense, nutrition, transportation, reproduction and housing. Interacting in two or more of these categories has a stabilizing effect on a symbiosis. Finding a new partner to replace a single function could happen fairly frequently in nature, but encountering a new partner to fulfill multiple functions would occur substantially less frequently.

Numerous economic theories have been applied to explain the evolution of stability in mutualisms. Partner choice game theory models include signaling, screening, partner fidelity feedback and host sanctions (reviewed in Archetti, Scheuring, *et al*. 2011). Partner choice can occur before establishing interactions between the players. If information about the players is asymmetric, the characteristics of interest can be hidden when the host selects its symbionts. This may be resolved through signaling, in which the more informed player may use a costly signal to accurately reveal its status. Alternatively, it can be resolved by screening, in which the less informed player imposes a costly barrier to entry that the more informed player accepts or rejects (Archetti, Ubeda, *et al*. 2011). As previously mentioned, once a symbiotic association has been established, it is critical for the host to continue maintaining cooperative symbionts. This has been described as the problem of hidden actions, which may be mediated by either partner fidelity feedback or host sanctions. In partner fidelity feedback fitness gains by both host and symbiont select against the persistent of cheaters in the population. In contrast, the host sanction model hypothesizes that penalties against cheaters prevent them from dominating symbiont populations (Bull & Rice 1991). Weyl and colleagues conducted empirical tests of these models, finding support for partner fidelity feedback, but not for host sanctions (2010).

Until quite recently, the majority of the literature on evolution of cooperation focused on stability of two player systems (*c.f.* Axelrod & Hamilton 1981; Bull & Rice 1991; Doebeli & Hauert 2005). Little and Currie (2009) hypothesized that a common parasitic "enemy" may serve to unit multiple players behind a common goal and reinforce cooperation between them. In addition, these authors present stable theoretical game-theory models of the prisoner's dilemma taking into account more than two players. Cooperation between many players in the face of adversity appears common in the natural world. This work brings the theory in line with what researchers observe in many mutualisms in the field.

II. Leaf-cutting ant-*Pseudonocardia* symbiosis as a model to study beneficial microbes

Fungus-growing ants engage in agriculture, farming a basidiomycetous fungal cultivar (genus *Leucoagaricus*). This ant-fungus-growing symbiosis originated approximately 50 million years ago (Schultz & Brady 2008). Researchers speculate the symbiosis originated with ants feeding on a free-living fungus. Over time the ants began to tend this fungus—slowly adopting an agricultural role, bringing bits of forage material to garden. Eventually an obligate mutualism developed in which the two players became completely dependent on one another for survival. Today the diversity of this monophyletic clade includes over 240 described species of fungus-growing ants in 13 genera, including two genera of leaf-cutting ants, the most derived members of this group (Schultz & Brady 2008). Across their diversity fungus-growing ants employ a variety of agricultural systems. Here, I focus on the leaf-cutting ants. These ants belong to two genera, *Atta* and *Acromyrmex*. Their range extends from the Southern US south to northern Argentina. Leaf-cutters are dominant herbivores in these areas, cutting between 12-17% of all leaves produced in Neotropical forests (Hölldobler & Wilson 1990). It was originally assumed

that the ants were eating these leaves, however, in 1874 Thomas Belt discovered that the ants were not consuming their leaves directly, but instead using them to manure their fungal garden. In exchange the fungus produced gongylidia, carbohydrate rich hyphal swellings on which the ants feed (Weber 1972). Farming allows the ants to harness plant resources inaccessible to many other herbivorous insects. The ants' fungal garden performs the role of an external digestive system, enabling the success of this mutualism.

Behavioral Ecology of Leaf-cutter ants. Leaf-cutter ants adapt their foraging strategies to their environments. For example, researchers documented *Atta colombica* harvesting leaf fragments more evenly from a larger number different plant species in an established forest community compared with a young forest community (Shepherd 1985). While the leaf-cutter ants utilize material from a wide variety of plants, these ants avoid cutting certain species; ants cut from only 142 of more than 500 species in the aforementioned study. Research with cycloheximide, a fungicide the ants cannot detect, has shown that the ants will begin to reject treated leaf material within hours after initial leaf fragment incorporations into their garden. Both *Atta* and *Acromyrmex* ants will continue to avoid even untreated leaves from the previously poisoned species for weeks (Ridley *et al*. 1996; North *et al*. 1999; Herz *et al*. 2008). Saverschek and colleagues (2010) went on to show that the ants could learn this avoidance behavior, and that this learned behavior could persist up to 18 weeks after initial exposure. These studies indicated that the ants are able to learn new foraging preferences in a matter of hours, apparently mediated by their fungal symbiont (c.f. Herz *et al*. 2008).

The ants' role in fresh substrate preparation does not end when they return to the nest. The ants masticate cut leaf fragments and mix it with fecal droplets, prior to incorporating this new

substrate into the garden (Weber 1972). These fecal droplets have been shown to contain a cocktail of enzymes from the ants' fungal garden including carboxymethylcellulases, laccases, pectinases and proteases that assist in leaf-breakdown (Ronhede *et al*. 2004). In addition, Mangone and Currie (2007) demonstrated that the ants pre-treating leaf-fragments using actinobacteria.

Acromyrmex and *Atta* ants build large colonies whose members can number into the millions (up to 2 million in *Acromyrmex*, and 7 million in *Atta*) (Hölldobler & Wilson 1990). While ants in both genera of leaf-cutters are polymorphic, mature *Atta* ant colonies have many more castes. In a typical *Acromyrmex* nest there are two castes of workers—minima and majors. In contrast, *Atta* nests can include up to eight different castes of workers (Wilson 1983). All leaf-cutter ants practice polyethism, dividing the tasks within a colony according to both caste and age. For example, the largest castes specialize in nest defense while the smallest castes remain within the fungal garden performing colony maintenance tasks and caring for brood (Wilson 1980). Younger media and majors also remain in the garden until they reach approximately 25 days old. Once these larger castes are older they leave the garden to forage for leaves, cut them and return to the colony. As the ants age and their mandibles become dull, they switch tasks again, this time carrying leaves and keeping the trail clear for their sisters (Schofield *et al*. 2010). At the end of the ants' lives they tend their waste dump.

***Escovopsis* parasite.** The ant's fungal cultivar is frequently infected by a specialized parasite in the genus *Escovopsis*. This microfungus directly consumes the fungal garden, most often forms persistent infections that lead to reduced cultivar growth rates and less often nest desertion (Currie, Mueller, *et al*. 1999; Currie 2001; Reynolds & Currie 2004). Researchers confirmed

Escovopsis could be cultured from approximately two-thirds of leaf-cutter colonies tested, often from multiple strains of parasite (superinfection) (Currie 2001; Taerum *et al*. 2007). This parasite is horizontally transmitted (Currie, Mueller, *et al*. 1999). *Escovopsis* is a diverse monophyletic genus, which has only been reported in association with fungus-growing ant colonies (Currie *et al*. 2003; Taerum *et al*. 2010). Phylogenetic work suggests that while a particular clade of *Escovopsis* specialized on the gardens of leaf-cutter ants, that within this group *Escovopsis* is broadly virulent against the gardens of many species of *Acromyrmex* and *Atta* ants (Currie *et al*. 2003; Taerum *et al*. 2010).

The fungus itself does not appear to be able to successfully defend itself against *Escovopsis*. When ants are removed from the fungal garden, it is quickly overgrown and decimated by the parasite (Currie, Scott, *et al*. 1999). In addition, *in vitro* bioassays show similar overgrowth between many different combinations of *Escovopsis* strains paired against an array of *Acromyrmex* and *Atta* fungal gardens (Poulsen *et al*. 2010). However, Little and Currie (2009) suggest that the *Escovopsis* may play a critical role in stabilizing the many partners in the leaf-cutter ant symbiosis, presenting empirical evidence that the common threat of the *Escovopsis* parasite may function to increase cooperation between leaf-cutting ants, their exosymbiont *Pseudonocardia* bacteria and their mutualistic fungal garden to combat this common enemy.

The ants play an active role in pest management, using numerous behaviors as well as an additional symbiotic partner to combat potential microbial threats. Behaviorally, the smallest castes of minor workers remove individual invasive spores by licking them out of the fungal gardens (Currie & Stuart 2001). The ants then place these spores into their infrabuccal pockets, apparently to sterilize them and transport them to the dump (Little *et al*. 2006). The ants also use

their forelegs to apply antimicrobial secretions from their metapleural gland to their fungal garden and leaf substrate material (as well as to treating themselves and other nestmates) for protection (Fernandez-Marin *et al*. 2006). Larger castes (media or majors) also assist in fungal cleaning and maintenance—grooming individual strands of mycelia to remove stay spores. These larger workers also engage in weeding, or removing larger chunks of infected or older garden and transporting these pieces out of the healthy garden chambers to either internal dump chambers or external dump piles (Currie & Stuart 2001).

***Pseudonocardia-Acromyrmex* ant association.** Leaf-cutter ants host Actinobacteria in the genus *Pseudonocardia* that produces antibiotic compounds active against the parasite *Escovopsis* (Currie, Mueller, *et al*. 1999). In exchange for garden protection, the ants appear to provide this symbiotic bacteria with nourishment. *Pseudonocardia* is typically maintained on modified structures on the ant cuticle, and most genera of fungus-growing ants appear to provide the bacteria with nourishment through glandular secretions (Currie *et al*. 2006). Location of growth varies by ant genus, with most genera of fungus-growing ants carrying *Pseudonocardia* on their propleural plates. Several studies have shown evidence supporting *Pseudonocardia's* role in garden defense. Currie and colleagues (2003) established that *Acromyrmex* ants tending the garden carried greater quantities of *Pseudonocardia* and that bacterial growth increased on ants in subcolonies exposed to the parasite. Additional lines of evidence include bioassays, demonstrating that *Pseudonocardia* inhibits *Escovopsis* (Currie, Scott, *et al*. 1999; Poulsen *et al*. 2010; Cafaro *et al*. 2011). These studies establish that different strains vary in their specificity against different strains of *Escovopsis*. Research using *Acromyrmex* subcolonies has also documented experimental evidence that the presence of this mutualistic bacteria improves

outcomes in response *Escovopsis* infection (Currie *et al*. 2003). Additional subcolony studies have shown that the degree of inhibition in bioassays correlated with improved subcolony garden survival rates (Poulsen *et al*. 2010). While not yet established in leaf-cutters, chemistry of one *Pseudonocardia*-fungus-growing ant, *Apterostigma dentigerum* has been determined. The *Pseudonocardia* strain investigated here produces a novel antifungal agent, dentigerumycin, that selectively inhibits *Escovopsis* (Oh *et al*. 2009).

Involving *Pseudonocardia* in garden defense likely has several advantages. First, since the mutualist garden and parasite are both fungi, removing defensive compound production and limiting its application enables a broader range of antifungal targets. Second, the bacteria's generation time is shorter than the ants' own, this faster potential reaction time gives the symbiosis an evolutionary advantage over the ants alone when competing against both specialized parasites and other pests on the system. Third, horizontal gene transfer is much more prevalent among bacteria, potentially enabling faster adaptation through acquisition of new antibiotic producing gene clusters (Ginolhac *et al*. 2005; Jenke-Kodama *et al*. 2005).

Major workers from most species across the diversity of the leaf-cutter ant genus *Acromyrmex* carry visible populations of their *Pseudonocardia* exosymbiont (Currie, Scott, *et al*. 1999; Poulsen *et al*. 2002; Currie *et al*. 2003). *Acromyrmex* ants have intrinsic variation in *Pseudonocardia* load from full body coverage ("sheep") to more limited coverage restricted to the propleural plates (Poulsen, Bot, Currie, *et al*. 2003). Scanning electron microscope observations indicate that the ants eclose symbiont-free, acquiring visible *Pseudonocardia* 3-5 days post-eclosion (Poulsen, Bot, Currie, *et al*. 2003). Visual observations indicate that bacterial symbiont load increase with time, peaking at 10-25 days, then tapering to more limited

propleural plate coverage as the ants begin foraging activity (Poulsen, Bot, Currie, *et al*. 2003).

Morphological differences between these two genera of leaf-cutters appear to reflect differences within the ant-*Pseudonocardia* symbiosis. For example, ants in the genus *Atta* do not appear to carry externally visible loads of *Pseudonocardia*, nor do these species have the gland or tubercle structures though to provide nutritional support to this symbiont (Currie *et al*. 2006). However, it is possible that the abundance of this Actinobacteria on the ants' cuticle is simply reduced (visible symbiotic bacteria are rare in nature), present in a restricted caste for a limited period of development or has shifted to an alternative location on or within *Atta* ants. *Atta* and *Acromyrmex* workers appear to use distinctly different strategies to combat microbial infections. When challenged with *Escovopsis* and or *Metarhizium anisopliae* (an entomopathogen), *Atta* ants increase metapleural gland grooming rates, applying antimicrobial compounds to a larger array of targets to combat potential infections while *Acromyrmex* ants do not increase metapleural gland grooming. This difference may arise in part because the metapleural gland secretions inhibit the *Pseudonocardia* symbiont (Poulsen, Bot, & Boomsma 2003). *Pseudonocardia* exoskeletal coverage appears inversely correlated with the frequency of metapleural gland grooming behavior in response to pest species. Leaf-cutter ant species appear to make a behavioral trade-off between employing metapleural gland secretions and relying on *Pseudonocardia* produced compounds for defense (Fernandez-Marin *et al*. 2006).

Host-Symbiont recognition. Work suggests that leaf-cutter ants in the genus *Acromyrmex* can recognize their own bacterial symbiont and shows that the ants demonstrate a preference for *Pseudonocardia* native to their own colony over non-native strains from other colonies (Zhang *et al*. 2007). In choice experiments, the ants spent long periods antennating substrates prior to

making a selection (C.R. Currie, personal communication). Zhang and colleagues speculated that this recognition is likely to be based on chemical signatures (2007). Richard and colleagues demonstrated the existence and possible utility of such signatures from both leaf-cutters ants themselves as well as their fungal cultivar (2007a; 2007b). Poulsen and colleagues (2011) demonstrated that while *Acromyrmex* ants normally prefer strains of *Pseudonocardia* genetically closest to their own colony, those preferences could be manipulated through exposure over time. This flexible symbiont recognition system could be critical to maintaining the symbiosis during switches of symbionts over evolutionary time.

Additional symbionts. Metagenomic studies have shown a wide variety of bacteria consistently associated with the ants' fungal gardens. The ants' fungal garden appears to host numerous microbes apparently active in digestion, including species from the genus *Enterobacter* (Suen *et al*. 2010; Aylward *et al*. 2012) and one or more symbiotic nitrogen-fixing bacteria in the genera *Klebsiella* and *Pantoea* (Pinto-Tomas *et al*. 2009). Yeasts have also been identified in the ants' fungal garden (Carreiro *et al*. 1997; Middelhoven *et al*. 2003; Carreiro 2004). Many of these yeasts have been demonstrated to produce toxins with anti-fungal properties (Carreiro *et al*. 2002)(Carreiro *et al*. 2002). One recent study has shown an ability for these compound to inhibit growth of a specialized parasite on the garden, *Escovopsis* (Rodrigues *et al*. 2009).

In addition to the previously mentioned symbionts, the leaf-cutter ant symbiosis includes a black yeast parasite that lives on the ants' propleural plates and consumes the ants beneficial *Pseudonocardia* bacteria (Little & Currie 2007). In addition leaf-cutter ants themselves often host a common insect intracellular symbionts in the genus *Wolbachia* (Andersen *et al*. 2012). Earlier studies in *Acromyrmex octospinosus* ant have shown that most workers are infected, often

by multiple strains (Van Borm *et al*. 2001; Frost *et al*. 2010; Van Borm *et al*. 2003). As researchers continue to explore this complex symbiosis undoubtedly additional symbionts will continue to be revealed.

Utility of studying the Leaf-Cutting Ant-*Pseudonocardia* system. Researchers have been developing and refining the leaf-cutting ant symbiosis as a model system to study mutualisms for over a decade. This system has numerous advantages including the ability to maintain colonies in the lab for years, to use subcolonies as replicates, to manipulate the individual components of the symbiosis including switching the fungal garden and the ants' visible, culturable, non-obligate exosymbiont, *Pseudonocardia*. An expanding variety of ant genomes now includes two leaf-cutting ants (*Acromyrmex echinatior* and *Atta cephalotes*) (Nygaard *et al*. 2011; Suen *et al*. 2011) with additional representation of the diversity of fungus-growing ants (*Trachymyrmex* a higher attine, and *Apterostigma* a basal attine) currently being sequenced (S Nygaard personal communication; C Currie personal communication). Together these tools combine to make this an excellent system to study symbiosis.

III. Overview

In this dissertation I explore the role of specificity in the leaf-cutter ant-*Pseudonocardia* symbiosis. I hypothesize that specificity will play an important role in the maintenance of this mutualism on multiple levels. In Chapter 2, I investigate the breadth of the *Pseudonocardia*-fungus-growing ant symbiosis. This symbiosis has been extensively documented among *Acromyrmex* leaf-cutter ants, but is controversial in *Atta* leaf-cutter ants that lack visible exosymbionts. Specifically, I explore the possible impact of culturing technique on symbiont

isolation. This is particularly important since symbionts are notoriously difficult to culture. In Chapter 3, I examine host-imposed constraints on *Pseudonocardia* acquisition in *Acromyrmex* leaf-cutter ants, exploring specific transmission mechanisms. I expect to find evidence of specificity in both components and timing necessary for successful acquisition. In Chapter 4, I focus on the genes involved in establishment and maintenance of symbiosis by monitoring gene expression in *Acromyrmex echinatior* leaf-cutting ants, comparing ants normally colonized by their exosymbiont (holosymbionts) against ants prevented from normally acquiring their exosymbiont (aposymbionts). In Chapter 5, I delve into a synthesis of chapters one through four, examining how this work can foster generalizable knowledge about the large field of symbiosis and what theoretical first principles might be found within this work, focusing on specificity. I discuss the role of the genomics revolution on current work and its potential future impact on the field. Finally, I conclude with thoughts on future directions in symbiosis research.

IV. References

Altura MA, Stabb E, Goldman W, Apicella M, McFall-Ngai MJ (2011) Attenuation of host NO production by MAMPs potentiates development of the host in the squid-*vibrio* symbiosis. *Cellular microbiology*, **13**, 527–537.

Andersen SB (2011) Dynamics of ant-microbial interactions: Coevolution along the parasitism-mutualism continuum.

Andersen SB, Boye M, Nash DR, Boomsma JJ (2012) Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. *Journal of Evolutionary Biology*, **25**, 1340–1350.

Archetti M, Scheuring I, Hoffman M, *et al*. (2011) Economic game theory for mutualism and cooperation. *Ecology Letters*, **14**, 1300–1312.

Archetti M, Ubeda F, Fudenberg D, *et al*. (2011) Let the Right One In: A Microeconomic Approach to Partner Choice in Mutualisms. *American Naturalist*, **177**, 75–85.

Axelrod R, Hamilton WD (1981) The Evolution of Cooperation. *Science*, **211**, 1390–1396.

Aylward FO, Burnum KE, Scott JJ, *et al*. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus garden. *ISME J*.

Van Borm S, Wenseleers T, Billen J, Boomsma JJ (2001) *Wolbachia* in leafcutter ants: a widespread symbiont that

may induce male killing or incompatible matings. *Journal of Evolutionary Biology*, **14**, 805–814.

Van Borm S, Wenseleers T, Billen J, Boomsma J. (2003) Cloning and sequencing of wsp encoding gene fragments reveals a diversity of co-infecting *Wolbachia* strains in *Acromyrmex* leafcutter ants. *Molecular Phylogenetics and Evolution*, **26**, 102–109.

Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology*, **8**, 218–230.

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, **154**, 275–304.

Bull JJ, Rice WR (1991) Distinguishing Mechanisms for the Evolution of Cooperation. *Journal of theoretical biology*, **149**, 63–74.

Burke GR, Normark BB, Favret C, Moran NA (2009) Evolution and Diversity of Facultative Symbionts from the Aphid Subfamily Lachninae. *Applied and Environmental Microbiology*, **75**, 5328–5335.

Cafaro MJ, Poulsen M, Little AEF, *et al*. (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 1814–1822.

Carreiro SC (2004) *Sympodiomyces attinorum* sp. nov., a yeast species associated with nests of the leaf-cutting ant *Atta sexdens*. *International Journal of Systemic and Evolutionary Microbiology*, **54**, 1891–1894.

Carreiro SC, Pagnocca FC, Bacci M, *et al*. (2002) Occurrence of killer yeasts in leaf-cutting ant nests. *Folia Microbiologica*, **47**, 259–262.

Carreiro SC, Pagnocca FC, Bueno OC, *et al*. (1997) Yeasts associated with nests of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. *Antonie van Leeuwenhoek*, **71**, 243–248.

Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*, **34**, 41–58.

Chun CK, Troll JV, Koroleva I, *et al*. (2008) Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-*vibrio* association. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11323–11328.

Currie CR (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, **128**, 99–106.

Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 7998–8002.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science*, **311**, 81–83.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.

Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **268**, 1033–1039.

Currie CR, Wong B, Stuart AE, *et al*. (2003) Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, **299**, 386–388.

Doebeli M, Hauert C (2005) Models of cooperation based on the Prisoner's Dilemma and the Snowdrift game. *Ecology Letters*, **8**, 748–766.

Dyall SD (2004) Ancient Invasions: From Endosymbionts to Organelles. *Science*, **304**, 253–257.

Fernandez-Marin H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 1689–1695.

Frank SA (1996) Host-Symbiont Conflict over the Mixing of Symbiotic Lineages. *Proceedings of the Royal Society B: Biological Sciences*, **263**, 339–344.

Frost CL, Fernandez-Marin H, Smith JE, Hughes WOH (2010) Multiple gains and losses of *Wolbachia* symbionts across a tribe of fungus-growing ants. *Molecular ecology*, **19**, 4077–4085.

Genkai-Kato M, Yamamura N (1999) Evolution of mutualistic symbiosis without vertical transmission. *Theoretical population biology*, **55**, 309–323.

Ginolhac A, Jarrin C, Robe P, *et al*. (2005) Type I polyketide synthases may have evolved through horizontal gene transfer. *Journal of Molecular Evolution*, **60**, 716–725.

Herz H, Hoelldobler B, Roces F (2008) Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. *Behavioral Ecology*, **19**, 575–582.

Hölldobler B, Wilson EO (1990) *The Ants*. Belknap Press of Harvard, Cambridge, MA.

Hooper LV, Gordon JI (2001) Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenicity. *Glycobiology*, **11**, 1R–10R.

Jenke-Kodama H, Sandmann A, Muller R, Dittmann E (2005) Evolutionary implications of bacterial polyketide synthases. *Molecular biology and evolution*, **22**, 2027–2039.

Little AEF, Currie CR (2007) Symbiotic complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biology Letters*, **3**, 501–504.

Little AEF, Currie CR (2009) Parasites may help stabilize cooperative relationships. *BMC Evolutionary Biology*, **9**, 124.

Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. *Biology Letters*, **2**, 12–16.

Login FH, Balmand S, Vallier A, *et al*. (2011) Antimicrobial Peptides Keep Insect Endosymbionts Under Control. *Science*, **334**, 362–365.

MacDonald SJ, Thomas GH, Douglas AE (2011) Genetic and metabolic determinants of nutritional phenotype in an insect-bacterial symbiosis. *Molecular ecology*, **20**, 2073–2084.

Mackey D, McFall AJ (2006) MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Molecular microbiology*, **61**, 1365–1371.

Mangone DM, Currie CR (2007) Garden substrate preparation behaviours in fungus-growing ants. *Canadian Entomologist*, **139**, 841–849.

Margulis L (1981) *Symbiosis in Cell Evolution*. Freeman, New York.

Martens EC, Goodrich-Blair H (2005) The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. *Cellular Microbiology*, **7**, 1723–1735.

Martens EC, Heungens K, Goodrich-Blair H (2003) Early Colonization Events in the Mutualistic Association between *Steinernema carpocapsae* Nematodes and *Xenorhabdus nematophila* Bacteria. *Journal of Bacteriology*, **185**, 3147–3154.

McFall-Ngai MJ (2002) Unseen forces: The influence of bacteria on animal development. *Developmental biology*, **242**, 1–14.

McFall-Ngai MJ (2007) Adaptive immunity - Care for the community. *Nature*, **445**, 153.

Medina M, Sachs JL (2010) Symbiont genomics, our new tangled bank. *Genomics*, **96**, 377–378.

Middelhoven WJ, Fonseca A, Carreiro SC, Pagnocca FC, Bueno OC (2003) *Cryptococcus haglerorum*, sp. nov., an anamorphic basidiomycetous yeast isolated from nests of the leaf-cutting ant *Atta sexdens*. *Antonie van Leeuwenhoek*, **83**, 167–174.

Moran NA (2006) Symbiosis. *Current Biology*, **16**, R866–R871.

North R, Jackson C, Howse P (1999) Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens rubropilosa*, regarding choice of substrate for the fungus. *Physiological Entomology*, **24**, 127–133.

Nygaard S, Zhang G, Schiott M, *et al*. (2011) The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. *Genome research*, **21**, 1339–1348.

Nyholm SV (2000) From the Cover: Establishment of an animal-bacterial association: Recruiting symbiotic *vibrios* from the environment. *Proceedings of the National Academy of Sciences*, **97**, 10231–10235.

Nyholm S, Mcfall-Ngai M (2004) The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nature Reviews Microbiology*, **2**, 632–642.

Oh D-C, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature chemical biology*, **5**, 391–393.

Pellmyr O, Huth CJ (1994) Evolutionary Stability of Mutualism between Yuccas and Yucca Moths. *Nature*, **372**, 257–260.

Pinto-Tomas AA, Anderson MA, Suen G, *et al*. (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science (Washington)*, **326**, 1120–1123.

Poulsen M, Bot ANM, Boomsma JJ (2003) The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. *Naturwissenschaften*, **90**, 406–409.

Poulsen M, Bot ANM, Currie CR, Boomsma JJ (2002) Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in *Acromyrmex* leaf-cutting ants. *Insectes Sociaux*, **49**, 15–19.

Poulsen M, Bot ANM, Currie CR, Nielsen MG, Boomsma JJ (2003) Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Functional Ecology*, **17**, 260–269.

Poulsen M, Cafaro MJ, Erhardt DP, *et al*. (2010) Variation in *Pseudonocardia* antibiotic defence helps govern

parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. *Environmental Microbiology Reports*, **2**, 534–540.

Poulsen M, Maynard J, Roland DL, Currie CR (2011) The role of symbiont genetic distance and potential adaptability in host preference towards *Pseudonocardia* symbionts in *Acromyrmex* leaf-cutting ants. *Journal of Insect Science*, **11**, 120.

Reynolds HT, Currie CR (2004) Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia*, **96**, 955–959.

Richard F-J, Poulsen M, Drijfhout F, Jones G, Boomsma JJ (2007) Specificity in chemical profiles of workers, brood and mutualistic fungi in *Atta*, *Acromyrmex*, and *Sericomyrmex* fungus-growing ants. *Journal of chemical ecology*, **33**, 2281–2292.

Richard F-J, Poulsen M, Hefetz A, *et al*. (2007) The origin of the chemical profiles of fungal symbionts and their significance for nestmate recognition in *Acromyrmex* leaf-cutting ants. *Behavioral Ecology and Sociobiology*, **61**, 1637–1649.

Ridley P, Howse P, Jackson C (1996) Control of the behaviour of leaf-cutting ants by their "symbiotic" fungus. *Experientia*, **52**, 631–635.

Rodrigues A, Cable RN, Mueller UG, Bacci M, Pagnocca FC (2009) Antagonistic interactions between garden yeasts and microfungal garden pathogens of leaf-cutting ants. *Antonie van Leeuwenhoek*, **96**, 331–342.

Ronhede S, Boomsma JJ, Rosendahl S (2004) Fungal enzymes transferred by leaf-cutting ants in their fungus gardens. *Mycological Research*, **108**, 101–106.

Saverschek N, Herz H, Wagner M, Roces F (2010) Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. *Animal Behaviour*, **79**, 689–698.

Schofield RMS, Emmett KD, Niedbala JC, Nesson MH (2010) Leaf-cutter ants with worn mandibles cut half as fast, spend twice the energy, and tend to carry instead of cut. *Behavioral Ecology and Sociobiology*, **65**, 969–982.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5435–5440.

Shepherd JD (1985) Adjusting Foraging Effort to Resources in Adjacent Colonies of the Leaf- Cutter Ant, *Atta colombica*. *Biotropica*, **17**, 245.

Silver AC, Rabinowitz NM, Kuffer S, Graf J (2007) Identification of *Aeromonas veronii*Genes Required for Colonization of the Medicinal Leech, Hirudo verbana. *Journal of Bacteriology*, **189**, 6763–6772.

Suen G, Scott JJ, Aylward FO, *et al*. (2010) An Insect Herbivore Microbiome with High Plant Biomass-Degrading Capacity. *Plos Genetics*, **6**, e1001129.

Suen G, Teiling C, Li L, *et al*. (2011) The Genome Sequence of the Leaf-Cutter Ant *Atta cephalotes* Reveals Insights into Its Obligate Symbiotic Lifestyle RID F-1026-2011. *Plos Genetics*, **7**, e1002007.

Taerum SJ, Cafaro MJ, Currie CR (2010) Presence of Multiparasite Infections Within Individual Colonies of Leaf-Cutter Ants. *Environmental Entomology*, **39**, 105–113.

Taerum SJ, Cafaro MJ, Little AEF, Schultz TR, Currie CR (2007) Low host-pathogen specificity in the leaf-cutting

ant-microbe symbiosis. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 1971–1978.

Thompson JN (1994) *The coevolutionary process*. University of Chicago Press, Chicago.

Weber NA (1972) *Gardening ants, the attines*. American Philosophical Society, Philadelphia.

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, **6**, 741–751.

Weyl EG, Frederickson ME, Yu DW, Pierce NE (2010) From the Cover: Economic contract theory tests models of mutualism. *Proceedings of the National Academy of Sciences*, **107**, 15712–15716.

Wilson EO (1980) Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *Behavioral Ecology and Sociobiology*, **7**, 143–156.

Wilson EO (1983) Caste and Division of Labor in Leaf-Cutter Ants (Hymenoptera, Formicidae, Atta): Colony Ontogeny of *Atta Cephalotes*. *Behavioral Ecology and Sociobiology*, **14**, 55–60.

Wollenberg MS, Ruby EG (2009) Population Structure of *Vibrio fischeri* within the Light Organs of *Euprymna scolopes* Squid from Two Oahu (Hawaii) Populations. *Applied and Environmental Microbiology*, **75**, 193–202.

Zhang MM, Poulsen M, Currie CR (2007) Symbiont recognition of mutualistic bacteria by *Acromyrmex* leaf-cutting ants. *ISME Journal*, **1**, 313–320.

CHAPTER 2. OPTIMIZATION OF ISOLATION METHODS SUGGESTS THE PRESENCE OF AN ASSOCIATION BETWEEN *PSEUDONOCARDIA* SYMBIONTS AND *ATTA* LEAF-CUTTING ANTS

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I. ABSTRACT

Fungus-growing ants associate with multiple symbiotic microbes, including Actinobacteria for the production of antibiotics. The best studied of these bacteria are within the genus *Pseudonocardia*, which in most fungus-growing ants is maintained visibly on the cuticle of the ant body. However, fungus-growing ants in the genus *Atta* do not have populations of Actinobacteria on their cuticle, making it unclear if *Atta* still engages in the symbiosis with *Pseudonocardia*. Here I explore whether optimizing culturing techniques can allow for successful isolation of *Pseudonocardia* from *Atta cephalotes* leaf-cutting ants. I obtained *Pseudonocardia* from nine of eleven culture-dependent isolation methods from all colonies sampled. The most efficient technique was bead-beating workers in phosphate buffer solution and subsequently plating the suspension on carboxymethylcellulose medium. I placed these strains in a global fungus-growing ant-associated *Pseudonocardia* phylogeny, revealing that while some strains grouped with clades of *Pseudonocardia* symbionts associated with other genera of fungus-growing ants, a large portion of the isolates localize in two novel phylogenetic clades previously not identified from fungus-growing ants. Our findings suggest that *Pseudonocardia* is likely associated with fungus-growing ants in the genus *Atta*, apparently

internalized over the course of the association, and that work localizing the symbiont and exploring its role in the ant genus is necessary to shed further light on this association.

II. INTRODUCTION

The fungus-growing ant-microbe symbiosis originated approximately 50 million years ago (Schultz & Brady 2008). Fungus-growing ants farm a specialized fungal cultivar, which serves as the ants' primary food source (Hölldobler & Wilson 1990). These fungal gardens host potentially virulent microfungal parasites in the genus *Escovopsis,* which consume the fungal mutualist (Currie, Mueller, *et al*. 1999; Reynolds & Currie 2004). To help suppress *Escovopsis* the ants associate with Actinobacteria in the genus *Pseudonocardia* that provide protection through antibiotic production (Currie, Scott, *et al*. 1999; Currie *et al*. 2003; Oh *et al*. 2009; Poulsen *et al*. 2010). Other Actinobacteria have been isolated or detected from fungus-growing ants using culture-independent methods (Van Borm *et al*. 2002; Kost *et al*. 2007; Haeder *et al*. 2009; Sen *et al*. 2009; Barke *et al*. 2010). Based on *in vitro* bioassay inhibition assays, these Actinobacteria have been suggested to also contribute to *Escovopsis* suppression (Haeder *et al*. 2009). However, evidence that additional Actinobacteria are specific symbionts with fungus-growing ants, as well as *in vivo* evidence of their functional role against *Escovopsis*, remains to be fully explored (c.f. Caldera & Currie in press; Cafaro *et al*. 2011).

The ant-*Pseudonocardia* association has been found across most of the phylogenetic diversity of fungus-growing ants (tribe Attini), including the paleo-attine genera *Apterostigma*, *Mycocepurus* and *Myrmicrocrypta* (lower attines), and the neo-attine genera *Cyphomyrmex*, *Mycetosoritis*, *Mycetarotes* (lower attines), *Trachymyrmex* and *Acromyrmex* (higher attines) (Currie, Scott, *et*

al. 1999; Currie *et al*. 2006; Cafaro *et al*. 2011). *Pseudonocardia* is typically maintained on modified structures on the ant cuticle, and most genera of fungus-growing ants appear to provide the bacteria with nourishment through glandular secretions (Currie *et al*. 2006). Location of growth varies by ant genus, with most genera carrying *Pseudonocardia* on the propleural plates. In at least two genera, *Sericomyrmex* and *Atta*, the abundance of Actinobacteria on the cuticle of the ants is markedly reduced, or completely absent, possibly as a consequence of a switch towards alternative defenses playing a more prominent role in controlling *Escovopsis* (Currie & Stuart 2001; Fernandez-Marin *et al*. 2009; see also Yek *et al*. 2012). Despite the absence of visible *Pseudonocardia* on the cuticle of *Atta* workers, isolates have previously been obtained and placed phylogenetically in the same clades as symbionts isolated from the other leaf-cutting ant genus *Acromyrmex* (clades IV and VI in Cafaro *et al*. 2011). Co-occurrence in these clades suggests that *Atta* and *Acromyrmex* maintain the same *Pseudonocardia* species; however, consistent isolations of *Pseudonocardia* from *Atta* have proven difficult, making it uncertain whether *Atta* persistently harbors symbiotic *Pseudonocardia*.

Bacterial symbionts are notoriously difficult to isolate (Moran 2006). This is often due to physiological dependence on host-provisioned nutrition, as in the pea aphid-*Buchnera* symbiosis (Darby *et al*. 2005). Consequently, it is possible that nutritional dependency is responsible for the difficulty in culturing *Pseudonocardia* from *Atta*. Optimizing culturing techniques might therefore determine whether *Pseudonocardia* is consistently present in *Atta*. Here I explore whether different isolation techniques can reliably acquire isolates of *Pseudonocardia* from *Atta* fungus-growing ants. I conduct targeted isolations for Actinobacteria using 11 methods, including bead beating, scraping, homogenization, and washing techniques. I apply these

methods to material from different colony components, including young and mature fungus garden, callow and mature workers, and dissected ant infrabuccal pockets.

III. MATERIALS & METHODS

ANT COLONIES

Most isolation attempts were done using five *Atta cephalotes* colonies collected in Gamboa, Panama in 2003 and 2005. These colonies (Pan03MB, Pan03BB, CC031208-10, AL050513-21, AL050513-22) had been housed at the University of Wisconsin-Madison for between 2 and 7 years at the time of isolation. Colonies were maintained at 24°C in the dark to mimic underground conditions, with overhead lights only illuminated periodically. Ant colonies were housed in plastic containers, with each colony kept in a large outer container (28cm H x 40cm W x 56cm L) accommodating one smaller plastic container for refuse material (dump) and one to five smaller plastic containers (ranging from 3.0 cm high by 7.5 cm x 7.5 cm to 11 cm high by 19.5 cm long by 12.5 cm wide) enclosing the colony fungus gardens. Each container had a 1 cm diameter hole drilled to allow ants to move in and out. Mineral oil was regularly applied to the top 4 cm of each outer container to prevent the ants from escaping. The ants were provisioned with maple (*Acer* sp.) and oak (*Quercus* sp.) leaves (frozen in the winter months and fresh during summer) three times per week. Leaves were supplemented with oatmeal, rice and cornmeal. Wet cotton balls were applied to the outer box to increase humidity. After initially determining what methods of isolation were most likely to be successful (see below), I increased the sampling to other laboratory colonies (*A. cephalotes* JS090511-01 from Panama; AP061022-01 from Costa Rica; EC090907-05, and MP090907-15 from Peru; *A. colombica* MP010708F-1 from Panama; *Atta* sp. EC090827-04 and UP08 from Peru).

ISOLATIONS

To compare the yield and efficiency of various Actinobacteria isolation techniques, I tested 11 different methods, using two types of media and materials from five *A. cephalotes* colonies including callow and media workers, infrabuccal pockets dissected from media workers, as well as young and mature garden. The isolation methods included washing, scraping, homogenization, sonication, and bead beating (see below and Table 1 for details). I performed five replicates for each method, for a minimum of 25 plates per method employed. In total, I conducted targeted isolations on 375 plates. For methods generating liquid inocula, plates were allowed to dry before being wrapped with parafilm.

Inocula were plated onto either chitin or carboxymethylcellulose (CMC) minimal nutrient media. Chitin medium is selective for Actinobacteria and is made by autoclaving 15 g agar, 3 g chitin, 0.575 g K_2HPO_4, 0.375 g $MgSO_4$ x $7H_2O$, 0.275 g KH_2PO_4, 0.0075 g $FeSO_4$ x $7H_2O$, 0.00075 g $MnCl_2$ x $4H_2O$, 0.00075 g $ZnSO_4$ x $7H_2O$ in 750 mL H_2O for 30 minutes. On this medium Actinobacteria appear white and faintly fuzzy, and grow flat on the surface. CMC media consisted of carboxymethylcellulose (5 g/L) and agar (15g/L) as the sole nutrient sources. Once bacteria were observed on minimal media, individual cultures were transferred to richer yeast malt extract agar (YMEA, 4 g yeast extract, 10 g malt extract, 4 g dextrose, 20 g agar, 1 L H_2O). Antifungals (20 ml/L nystatin and 0.05 g/L cycloheximide) were added to all media (Cafaro & Currie 2005; Zhang *et al*. 2007).

Actinobacteria derived from washes were obtained by aseptically placing workers in 1 ml of autoclaved Milli-Q water inside an autoclave-sterilized 1.5 ml microcentrifuge tube, which was

vortexed for 30 seconds. One hundred μl of this suspension were plated onto each of five plates. Using this washing technique, I sampled medium-sized workers (three), callow workers (two, identified by their lighter color), dissected infrabuccal pockets, and small pieces of top (youngest) or middle (older) garden material. Media workers and middle fungus garden were sonicated for 45 seconds prior to plating.

'Scraping' isolations were done by rubbing the surface of workers with a sterilized bent micro-spatula under a dissecting microscope. This method has been employed to isolate visible *Pseudonocardia* from the cuticle of workers from other fungus-growing ant genera (Cafaro & Currie 2005). Bacteria inocula from the tool surface were transferred to chitin plates. This method was conducted on the cuticle of medium-sized workers.

Infrabuccal pocket material was collected by dissecting out pockets, then using a sterile isolation loop to spread the pocket content directly onto chitin plates (Little *et al*. 2006).

Bead beating (Mini-Beadbeater-96, BioSpec products http://www.biospec.com) was performed on single medium-sized ants placed in 1.5 ml microcentrifuge tubes containing 500 ml phosphate buffer solution (PBS). I used PBS for bead beating following Medina-Rivera (2008), who successfully used this solution to isolate Actinobacteria from a non fungus-growing ant, *Odontomachus ruginodis*. Ants were bead beaten for two and a half minutes, after which 100 μl of the suspension were pipetted onto either chitin or CMC plates.

In order to evaluate whether Actinobacteria could have been introduced to the colonies on lab-provisioned leaves, I isolated for Actinobacteria from leaf material. These isolations targeted both external bacteria from leaf surfaces and possible endophytic Actinobacteria from surface-

sterilized leaves. Two to three leaves were collected from each of two oak and two maple trees routinely used to feed the ants in Madison, WI, for a total of five leaves per species and 10 leaves in total. Leaves were placed in sterile tubes and transported back to the lab for processing. I cut leaves into 0.5 cm^2 pieces, and performed four types of targeted isolations. One set of untreated leaves was blotted directly onto chitin plates to isolate external bacteria. A second set was homogenized for 20 seconds with a sterile plastic pestle in 1.5 ml Eppendorf tubes containing 500 ml autoclaved Milli-Q water. One hundred μl of liquid were then plated onto chitin plates, spread and allowed to dry before wrapping with parafilm. A third set of 0.5 cm^2 pieces were surface sterilized by submersion in 70% ethanol for 2 minutes. Pieces were then submerged in 1% Bleach containing 0.001% Tween 20 for 1 minute. Leaves were subsequently rinsed in 1 mL autoclaved Milli-Q water for at least 10 seconds. The bleach wash was repeated three times. Finally, these surface-sterilized pieces were homogenized and plated as described above. A fourth set of 0.5 cm^2 pieces were surface sterilized as described above and blotted directly onto chitin plates to assess the efficiency of surface sterilization.

In all cases, plates were incubated for 3–5 weeks at room temperature (~22°C) before bacterial colonies with Actinobacteria morphology (filamentous, dry and spiky or dusty in appearance) were picked and transferred to YMEA. I allowed these colonies to grow for 1-4 weeks, and then confirmed morphology-based species identifications for all *Pseudonocardia*-like colonies using 16S rRNA sequencing (see below for details).

To compare and quantify the efficiency of Actinobacteria and *Pseudonocardia* isolation, I counted the number of colony forming units (CFUs) produced on plates. I further calculated the

proportion of colonies from which I was able to successfully isolate Actinobacteria and *Pseudonocardia* for each method. Additional *Pseudonocardia* isolated from lab colonies were included in tables and the phylogenetic analysis but not in the method efficacy analysis.

DNA SEQUENCING AND PHYLOGENETIC ANALYSIS

Once bacteria were obtained in pure culture I extracted DNA using a standard cetyltrimethylammonium bromide (CTAB) protocol (Poulsen *et al*. 2005; Cafaro & Currie 2005). DNA was quantified using a NanoDrop photospectrometer (Thermo-Fisher) and diluted to 50 ng/μl in tris-ethylenediaminetetraacetic acid (TE) buffer. I then amplified 16S ribosomal RNA using universal primers (27f 5'AGAGTTTGATCMTGGCTCAG'3 and 1492r 5'TACGGYTACCTTGTTACGACTT'3, Giovannoni 1991) and nuclear Elongation Factor-Tu (EF-Tu if 5'CACGACAAGTCCCGAACCT'3 and ir 5'AGTTGTTGAAGAACGGGGTG'3) (Caldera & Currie *in press*). Amplicons were sequenced using the dideoxyterminator method (Big Dye terminator mix; Applied Biosystems, Foster City, CA) on an ABI 3730xl DNA Analyzer at the University of Wisconsin Biotechnology Center (Madison, WI) following a cleaning using the CleanSeq magnetic bead protocol (Agencourt Biosciences, Beverly, MA).

I edited sequences using Sequencher 4.5 (Gene Codes Corporation) to assemble the forward and reverse sequences, then reconciled differences and removed automated calling errors as required. In cases of uncertainty, samples were re-sequenced. To identify each isolate to the genus level, I used BLASTn nucleotide sequences against the non-redundant nucleotide collection using megablast (Morgulis *et al*. 2008). I screened all 16S sequences for possible chimeras using Bellerophon (Huber *et al*. 2004) through the Greengenes database with the default parameters. I

found no chimeric sequences.

To generate a phylogeny for all *Pseudonocardia* isolated, I aligned sequences from the EF-Tu gene using Clustal X (http://www.clustal.org/), checked the alignment in MacClade 4.07 (Maddison & Maddison 2005) and thereafter used Mega 5.0 (Tamura *et al*. 2007) to generate a Maximum likelihood phylogeny (Tamura-Nei assuming uniform rates and using all sites, sequences were truncated such that all were the same length) and bootstrapped 1000 times. The analysis included 38 strains previously isolated and sequenced from other colonies of fungus-growing ants (14 from leaf-cutting ants: 5 in genus *Atta*, and 9 in the genus *Acromyrmex*) as well as 16 species of free-living (non-ant-associated) *Pseudonocardia*. I rooted the tree using two species of *Streptomyces*. All generated sequences are available at GenBank under accession numbers JQ731834-85.

IV. RESULTS

I was consistently able to isolate Actinobacteria from *Atta* colonies using the different methods employed (Table 2). On average, across all isolation methods and ant colonies, I obtained 20.6±6.3 (Mean±SE) Actinobacterial CFUs per plate. I also isolated *Pseudonocardia* from all *Atta* colonies, with an average of 2.86±1.47 CFUs of *Pseudonocardia* per plate across all isolation methods and the five ant colonies sampled.

For Actinobacteria isolations, bead beating was the most successful method, generating an average of 40.1±24.0 (Mean±SE) CFUs of Actinobacteria per plate on chitin medium and 35.8±24.9 CFUs per plate on CMC medium. The washing method also resulted in large numbers of Actinobacteria, including an average of 16.4±10.1, 11.2±7.7, and 32.4±27.9 CFUs from

washed workers, sonicated workers, and sonicated mature garden, respectively. Further, worker scrapes, worker infrabuccal pockets scrapes, and worker infrabuccal pocket washes produced Actinobacterial CFUs, an average of 16.2±11.6, 32.0±11.3, and 48.6±17.4 per plate, respectively. These differences in Actinobacterial CFUs isolated by each of the 11 methods were significantly different by the Kruskal-Wallis rank sum test (df =10, p = 0.008091). Bead beating, worker scraping, and infrabuccal pocket washes all resulted in the isolation of Actinobacteria, while worker washes and infrabuccal pocket scrapes were less efficient, yielding Actinobacteria from only 4 of the 5 colonies. Worker washes with sonication and mature garden washes yielded Actinobacteria from 3 of the 5 colonies, while mature garden washes with sonication and young garden washes resulted in strains from only 2 of the 5 colonies. Washing callow workers was the only method that did not yield any Actinobacteria.

For *Pseudonocardia* isolations, nine of our eleven isolation methods resulted in at least one isolate per colony (Table 2). Bead-beating workers was also the most efficient method for obtaining *Pseudonocardia*, yielding 7.4±5.1 and 4.3±3.3 CFUs per plate on CMC and chitin, respectively. Bead beating isolated significantly more CFUs of *Pseudonocardia* than the other methods tested here by the Wilcoxon Rank sum test (W = 127, p-value = 0.01564). However, this method was not successful across all colonies, recovering *Pseudonocardia* from only 4 of the 5 colonies tested. Worker washing was the only other method that resulted in substantial *Pseudonocardia* CFUs, with an average of 6±2.8 per plate. Wilcoxon Rank sum test for this method was close to, but did not reach statistical significance (W = 68, p = 0.05589). Worker washes resulted in the isolation of *Pseudonocardia* from 3 of the 5 colonies tested (Table 2). Infrabuccal pocket scrape, worker scrape and bead beating from mature workers onto chitin

yielded few CFUs of *Pseudonocardia,* but did result in isolates from 2 of the 5 colonies sampled. Worker wash with sonication, mature garden wash, mature garden wash with sonication, and young garden washes generated isolates of *Pseudonocardia* in only one of the five colonies, yielding extremely low CFU counts (0.4±0.2). I was able to obtain *Pseudonocardia* from 10 additional lab colonies applying these methods (see Table 3, Figure 1).

Sequencing 16S rRNA genes of Actinobacteria obtained in this study revealed some phylogenetic diversity: seven *Streptomyces,* two *Nocardioides*, and seventeen *Pseudonocardia* isolates (Table 3). Because 16S rRNA genes alone provide limited species-level phylogenetic resolution, to further elucidate potential specificity I generated a phylogeny of the *Pseudonocardia* isolates based on EF-Tu (Figure 1). Three *Pseudonocardia* isolates grouped with isolates from *Acromyrmex* leaf-cutting ants (one in clade IV and two in clade VI, Figure 1), but I also recovered *Pseudonocardia* from two clades that had not previously been identified in associations with fungus-growing ants (Figure 1, clades labeled with circle and square, respectively). A concurrent study employing bead-beating with *Acromyrmex* ants identified sequences mapping to these novel clades from three *Acromyrmex* leaf-cutter ants [unpublished data, sequences included in Figure 1; *Acromyrmex echinatior* colonies AP061104-01, AP061105-02, and AP061106-01 from Costa Rica].

I did not isolate *Pseudonocardia* from oak and maple leaves blotted on chitin media, but did obtain CFUs of other Actinobacteria: an average of 0.4±0.28 (Mean±SE) (0.5±0.47 from oak, and 0.3± 0.28 from maple). Isolations by homogenization resulted in an average of 0 CFUs of Actinobacteria from oak, and 0.8±0.76 from maple. Sequencing of the strains recovered from

these isolations revealed only *Streptomyces*. No Actinobacteria colonies were isolated on CMC medium. The Actinobacteria isolation rate from leaves was therefore low overall, with only a single leaf of the ten resulting in CFUs.

V. DISCUSSION

I tested the efficiency of several methods for isolating Actinobacteria, in particularly *Pseudonocardia,* from *Atta cephalotes* leaf-cutting ants, and successfully isolated Actinobacteria with the majority of methods tested, including washing, scraping, and bead beating (Table 2). These Actinobacteria were from three genera: *Streptomyces, Nocardioides*, and *Pseudonocardia*. Bead beating workers in PBS and plating the supernatant on CMC plates resulted in the highest CFU counts, and produced *Pseudonocardia* from four out of five colonies. Washing workers was the next most efficient method, yielding *Pseudonocardia* from three of five colonies. All other methods yielded an average of less than one CFU of *Pseudonocardia* per Petri plate; however, worker scrapes and worker infrabuccal pocket scrapes were successful from two of five colonies even though at low CFU levels. Consequently, the most efficient methods for *Pseudonocardia* isolation involved direct processing of worker ants, while fungus garden isolations were less successful. Isolates of *Pseudonocardia* were never obtained from leaves provided to the colonies, despite endophytic species being known to occur within this bacterial genus (*e.g*. Chen *et al*. 2009, Nimnoi *et al*. 2009). Collectively, these findings support that *Atta* colonies consistently harbor *Pseudonocardia* and that this symbiotic bacterium is associated with the colony workers. This is congruent with the finding that the majority of the diversity of fungus-growing ants maintains a visible cover of Actinobacteria on the cuticle (*c.f*. Currie *et al*. 2006).

Previous work has had limited success isolating *Pseudonocardia* using maceration and vortexing methods, but *Pseudonocardia* had previously been obtained from five *Atta* colonies (Zhang *et al*. 2007; Cafaro *et al*. 2011). Bead beating represents a substantial improvement over previously employed techniques. This study isolated twenty additional strains of *Pseudonocardia* (4 confirmed by 16S rRNA gene sequencing, 3 confirmed by Ef-Tu gene sequencing and 13 confirmed by both) from a total of 15 colonies from three *Atta* species. Phylogenetic analysis of these strains placed one and two strains into clades IV and VI (Cafaro *et al*. 2011), respectively, alongside isolates from *Acromyrmex* leaf-cutting ants and the five strains previously isolated from *Atta* (Figure 1). Although *Atta* ants do not carry visible populations of their bacterial symbiont, the clustering of these strains suggests a specific, potentially long-term, association between *Atta* and *Pseudonocardia*. The remaining 13 strains of *Atta*-associated *Pseudonocardia* occur in two apparently novel clades that may represent hitherto unknown diversity within the symbiosis. These bacteria do not appear to be present on provisioned leaf material, suggesting their presence in the symbiosis, but I cannot rule out alternative sources of introduction of the bacteria to ant workers. These novel clades also include *Pseudonocardia* isolated from *Acromyrmex* leaf-cutter ants, which have so far only been found associated with clades IV and VI (Poulsen *et al*. 2005; Cafaro *et al*. 2011). While bead beating whole ants was very efficient in isolating *Pseudonocardia*, these bacteria could originate from the gut or another location within the ant. Additional isolations and phylogenetic studies will be needed to validate the presence of these novel genotypes and to further explore the extent and role of this relationship.

Our limited isolation success from certain colony components concurs with the culture dependent findings by Mueller and colleagues (2008). Both studies found little to no

Pseudonocardia from the majority of *Atta* colony components, including fungal gardens and the ants' infrabuccal pockets. Further, *Pseudonocardia* was not detected in a recent community metagenomic study of *Atta cephalotes* garden, suggesting that it is unlikely to reside in this component of the system (Aylward *et al*. 2012). Neither Mueller and colleagues (2008), Aylward and colleagues (2012) nor this study found convincing evidence of garden or other environmental sources for *Pseudonocardia* in the *Atta* leaf-cutting ant symbiosis. Mueller and colleagues (2008) did not attempt isolations directly from workers (except infrabuccal pockets), while I found the highest isolation rate directly from whole ants. Our findings support an association of *Pseudonocardia* with the system, and specifically with the ant workers, but not in the infrabuccal pocket.

Atta colonies include large work forces of highly polymorphic ants, in addition to age-dependent division of labor (Hölldobler & Wilson 1990). In other genera of attine ants, *Pseudonocardia* are present on a variety of cuticular structures (Currie *et al*. 2006), and it is possible that the symbiont is present on inaccessible parts of the ant cuticle, perhaps even in specific castes. Furthermore, it is conceivable that only a subset of worker castes associates with *Pseudonocardia* and employs its antifungal compound(s), as is the case in the less polymorphic *Acromyrmex* genus where major workers are the main carrier of *Pseudonocardia* (Poulsen *et al*. 2002; Currie *et al*. 2003). Collectively, this suggests that age- or caste dependent within-colony composition of the bacterial symbiont population could make it harder to obtain without considerable targeted effort to screen across a wide range of ages and castes.

It is well known that many symbiotic microbes are extremely difficult to culture in isolation, and

even when these microbes can grow in culture, they are often extremely fastidious, requiring a specific combination of growth conditions and nutrients normally provisioned by the host to meet their physiological needs (Darby & Welburn 2003). However, a considerable effort allowed us to isolate strains of *Pseudonocardia* from 15 *Atta* colonies included in this study. Currie and Stuart (2001) showed that *Atta* ants employ fungus grooming at increased levels in response to both generalist and specialist pathogens. Fernández-Marín and colleagues (2009) went on to demonstrate that *Atta* ants manifest increased levels of metapleural gland grooming behavior when challenged with *Escovopsis*. Together these behaviors may reduce *Atta* ants' need to utilize *Pseudonocardia* for defense. Consequently, although our work supports that there may still be an association between *Pseudonocardia* and *Atta*, further work is needed to identify the location of *Pseudonocardia* in *Atta*, as well as to deduce the role and impact of the bacteria on *Atta* colonies.

VI. ACKNOWLEDGEMENTS

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VII. REFERENCES:

Aylward FO, Burnum KE, Scott JJ, *et al*. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus garden. *ISME J*.

Barke J, Seipke RF, Grüschow S, *et al*. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biology*, **8**, 109.

Van Borm S, Billen J, Boomsma JJ (2002) The diversity of microorganisms associated with Acromyrmex leafcutter ants. *BMC evolutionary biology*, **2**, 9.

Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Canadian Journal of Microbiology*, **51**, 441–446.

Cafaro MJ, Poulsen M, Little AEF, *et al*. (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 1814–1822.

Caldera EJ, Currie CR (in press) The population structure of antibiotic-producing bacterial symbionts of *Apterostigma dentigerum* ants: Impacts of coevolution and multi-partite symbiosis. *American Naturalist*.

Chen HH, Qin S, Li J, *et al*. (2009) *Pseudonocardia endophytica* sp. nov., isolated from the pharmaceutical plant Lobelia clavata. *International Journal of Systematic and Evolutionary Microbiology*, **59**, 559–563.

Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos*, **101**, 91–102.

Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 7998–8002.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science*, **311**, 81–83.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.

Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **268**, 1033–1039.

Darby AC, Chandler SM, Welburn SC, Douglas AE (2005) Aphid-Symbiotic Bacteria Cultured in Insect Cell Lines. *Applied and Environmental Microbiology*, **71**, 4833–4839.

Darby AC, Welburn SC (2003) Symbiont Culture. In: *Insect Symbiosis* (eds Bourtzis K, Miller TA), pp. 141–156. CRC Press, Boca Raton, FL.

Fernandez-Marin H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT (2009) Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proceedings of the Royal Society B-Biological Sciences*, **276**, 2263–2269.

Haeder S, Wirth R, Herz H, Spiteller D (2009) From the Cover: Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis. *Proceedings of the National Academy of Sciences*, **106**, 4742–4746.

Hölldobler B, Wilson EO (1990) *The Ants*. Belknap Press of Harvard, Cambridge, MA.

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics*, **20**, 2317–2319.

Kost C, Lakatos T, Böttcher I, *et al*. (2007) Non-specific association between filamentous bacteria and fungus-growing ants. *Naturwissenschaften*, **94**, 821–828.

Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. *Biology Letters*, **2**, 12–16.

Maddison DR, Maddison WP (2005) *MacClade 4 release version 4.07 for OSX*. Sinauer Associates, Inc., Sunderland, Massachusetts.

Medina-Rivera M (2008) Isolation of Actinomycetes from the ant *Odontomachus ruginodis*.

Moran NA (2006) Symbiosis. *Current Biology*, **16**, R866–R871.

Morgulis A, Coulouris G, Raytselis Y, *et al*. (2008) Database indexing for production MegaBLAST searches. *Bioinformatics*, **24**, 1757–1764.

Mueller UG, Dash D, Rabeling C, Rodrigues A (2008) Coevolution between Attine ants and Acintomycete bacteria: A reevaluation. *Evolution*, **62**, 2894–2912.

Nimnoi P, Pongsilp N, Lumyong S (2009) Endophytic actinomycetes isolated from *Aquilaria crassna Pierre ex Lec* and screening of plant growth promoters production. *World Journal of Microbiology and Biotechnology*, **26**, 193–203.

Oh D-C, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature chemical biology*, **5**, 391–393.

Poulsen M, Bot ANM, Currie CR, Boomsma JJ (2002) Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in Acromyrmex leaf-cutting ants. *Insectes Sociaux*, **49**, 15–19.

Poulsen M, Cafaro M, Boomsma JJ, Currie CR (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of Acromyrmex leaf-cutting ants. *Molecular ecology*, **14**, 3597–3604.

Poulsen M, Cafaro MJ, Erhardt DP, *et al*. (2010) Variation in Pseudonocardia antibiotic defence helps govern parasite-induced morbidity in Acromyrmex leaf-cutting ants. *Environmental Microbiology Reports*, **2**, 534–540.

Reynolds HT, Currie CR (2004) Pathogenicity of Escovopsis weberi: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia*, **96**, 955–959.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5435–5440.

Sen R, Ishak HD, Estrada D, *et al*. (2009) Generalized antifungal activity and 454-screening of Pseudonocardia and Amycolatopsis bacteria in nests of fungus-growing ants. *Proceedings of the National Academy of Sciences*, **106**, 17805–17810.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular biology and evolution*, **24**, 1596–1599.

Yek SH, Boomsma JJ, Poulsen M (2012) Towards a Better Understanding of the Evolution of Specialized Parasites of Fungus-Growing Ant Crops. *Psyche: A Journal of Entomology*, **2012**, 1–10.

Zhang MM, Poulsen M, Currie CR (2007) Symbiont recognition of mutualistic bacteria by Acromyrmex leaf-cutting ants. *ISME Journal*, **1**, 313–320.

VIII. TABLES:

Table 1. Isolation Methods An overview of the Actinobacteria isolation methods used in this study. Techniques fall into three main categories: washing, scraping and beat beating. Within each method I varied the liquid used (water or PBS), growth media used (chitin or CMC), and presence/absence of sonication. Colony materials included whole callow and media workers, infrabuccal pockets isolated from media workers, as well as young and mature garden.

Technique	***Wash***		***Scrape***	***Bead Beat***	
Liquid	H_20	H_20	-	PBS	PBS
Media	chitin	chitin	chitin	chitin	CMC
Sonication	-	+	-	-	-
Starting Material (Method #)	Media workers Callow workers Infrabuccal pocket Young garden Mature garden	Media workers Mature garden	Media workers Infrabuccal pocket	Media workers	Media workers

Table 2. Isolation of total Actintobacteria and *Pseudonocardia* in particular from *Atta cephalotes* colonies. Colony forming units and isolation success rate of Actinobacteria and *Pseudonocardia* from the 11 methods. CFU counts and standard errors are reported per plate per colony, based on an average of five plates from each of five colonies, for the total Actinobacteria as well as for *Pseudonocardia* specifically. Success rates are reported per colony, representing the proportion of the colonies yielding at least one CFU of total Actinobacteria and *Pseudonocardia* in particular.

Method	**CFUs Mean±SE**	**Success rate**	**CFUs Mean±SE**	**Success rate**
Worker Wash	16.4±10.1	80%	6.0±2.8	60%
Worker Wash & Sonicate	11.2±7.7	60%	0.8±0.4	20%
Mature Garden Wash	1.2±0.5	60%	0.4±0.4	20%
Mature Garden Wash & Sonicate	32.4±27.9	40%	0.2±0.2	20%
Young Garden Wash	1.0±0.6	40%	0.4±0.4	20%
Callow Wash	0±0	0	0±0	0
Worker Infrabuccal pocket scrape	32.2±11.3	80%	0.4±0.2	40%
Worker Infrabuccal pocket wash	48.6±17.4	100%	0±0	0
Worker Scrape	16.2±11.6	100%	0.4±0.2	40%
Worker Beadbeat PBS Chitin	40.1±20.4	100%	4.3±3.3	40%
Worker Beadbeat PBS CMC	35.8±24.9	100%	7.4±5.1	80%

Table 3. Actinobacteria species (next page). Table of 16S rRNA sequences acquired from *Atta* sp. colonies and leaf material. Abbreviations are *A. cep*: *Atta cephalotes*; *A. col*: *Atta Colombia; Acer*: *Acer* sp.; *Q. pal*: *Quercus palustris*; Chi: Chitin; CMC: carboxymethylcellulose; Code: Colony Code; CR: Costa Rica. In the far let column, (A), (B) and (C) indicate different isolates from the same colony.

Table 3 (caption previous page)

Code (Isolate)	Taxon	Location	Method	Closest 16S BLAST hit (Genbank Accession number)	GenBank #
AL050513-21(A)	*A. cep*	Panama	worker scrape-Chi	*Streptomyces* sp. JS520 (JQ288109.1)	JQ731834
AL050513-21(B)	*A. cep*	Panama	worker infrabuccal pocket wash-H_2O/Chi	*Streptomyces* sp. RS-2011-I28 (HE617241.1)	JQ731835
AL050513-21(C)	*A. cep*	Panama	fungal garden wash/sonicate-H_2O/Chi	*Streptomyces drozdowiczii* strain GYB24 (JQ342930.1)	JQ731836
AL050513-21	*A. cep*	Panama	worker bead beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731837
AL050513-22(A)	*A. cep*	Panama	worker bead beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731838
AL050513-22(B)	*A. cep*	Panama	worker wash, worker bead beat-PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731839
AP061022-01(A)	*A. cep*	CR	worker bead beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731840
CC031208-10	*A. cep*	Panama	worker wash/sonicate-H_2O/Chi	*Nocardioides albus* (AF004997.1)	JQ731841
CC031208-10(A)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardia* sp. GB7 (EF451805.1)	JQ731842
CC031208-10(B)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardiaceae* isolate SR 244a (X87314.1)	JQ731843
EC090827-04	*A. cep*	Peru	worker bead beat-PBS/CMC	*Pseudonocardia* sp. MVT7 (EU931094.1)	JQ731844
EC090907-05	*A. cep*	Peru	worker bead beat-H_2O/Chi	*Pseudonocardia* sp. TFS 1235 (EF216360.1)	JQ731845
MP010708F-1	*A. col*	Panama	fungal garden wash-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ768367
MP090907-15	*A. cep*	Peru	worker bead beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731846
BB03	*A. cep*	Panama	worker infrabuccal pocket scrape-Chi	*Streptomyces* sp. 172624 (EF550508.1)	JQ731847
BB03(A)	*A. cep*	Panama	worker bead beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731848
BB03(B)	*A. cep*	Panama	worker bead beat-PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731849
MB03(A)	*A. cep*	Panama	worker wash/sonicate-H_2O/Cih	*Streptomyces* sp. Ank245 (HQ662223.1)	JQ731850
MB03(B)	*A. cep*	Panama	new garden wash-H_2O/Chi	Actinobacterium ZXY009 (JN049458.1)	JQ731851
MB03(C)	*A. cep*	Panama	worker wash/sonicate-H_2O/Chi	*Streptomyces* sp. 10213 (FJ262955.1)	JQ731852
MB03	*A. cep*	Panama	worker wash & wash/sonicate-H_2O/Chi	*Nocardioides albus* (AF004997.1)	JQ731853
MB03(A)	*A. cep*	Panama	worker bead beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731854
MB03(B)	*A. cep*	Panama	garden wash-H_2O/Chi	*Pseudonocardia* sp. CC011205-08 (EU928998.1)	JQ731855
MB03(C)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardia* sp. GB7 (EF451805.1)	JQ731856
UP08	*Atta* sp.	Peru	worker bead beat-PBS/Chi	*Pseudonocardia* sp. FXJ3.021 (JN683673.1)	JQ731857
JS090511-01	*A. cep*	Panama	worker bead beat-PBS/Chi	*Pseudonocardia* sp. JS020524-10_B1F6 (EU928990.1)	JQ731858
N/A (A)	*Q. pal*	US	blot-Chi	*Streptomyces glauciniger* strain FXJ14 (AY314782.1)	JQ731859
N/A (B)	*Q. pal*	US	blot-Chi	*Streptomyces* sp. I08A-01824 (GU550598.1)	JQ731860
N/A (C)	*Q. pal*	US	blot-Chi	*Streptomyces owasiensis*, strain: NBRC 13832 (AB184515.1)	JQ731861
N/A (A)	*Acer*	US	homogenize-H_2O/Chi	*Streptomyces* sp. 163005 (GU263862.1)	JQ731862
N/A (B)	*Acer*	US	homogenize-H_2O/Chi	*Streptomyces* sp. JW1 (EU906929.1)	JQ731863
N/A (C)	*Acer*	US	blot-Chi	*Streptomyces halstedii* strain G8A-5 16S (CP002993.1)	JQ731864

Figure 1. *Pseudonocardia* Phylogeny (Caption next page)

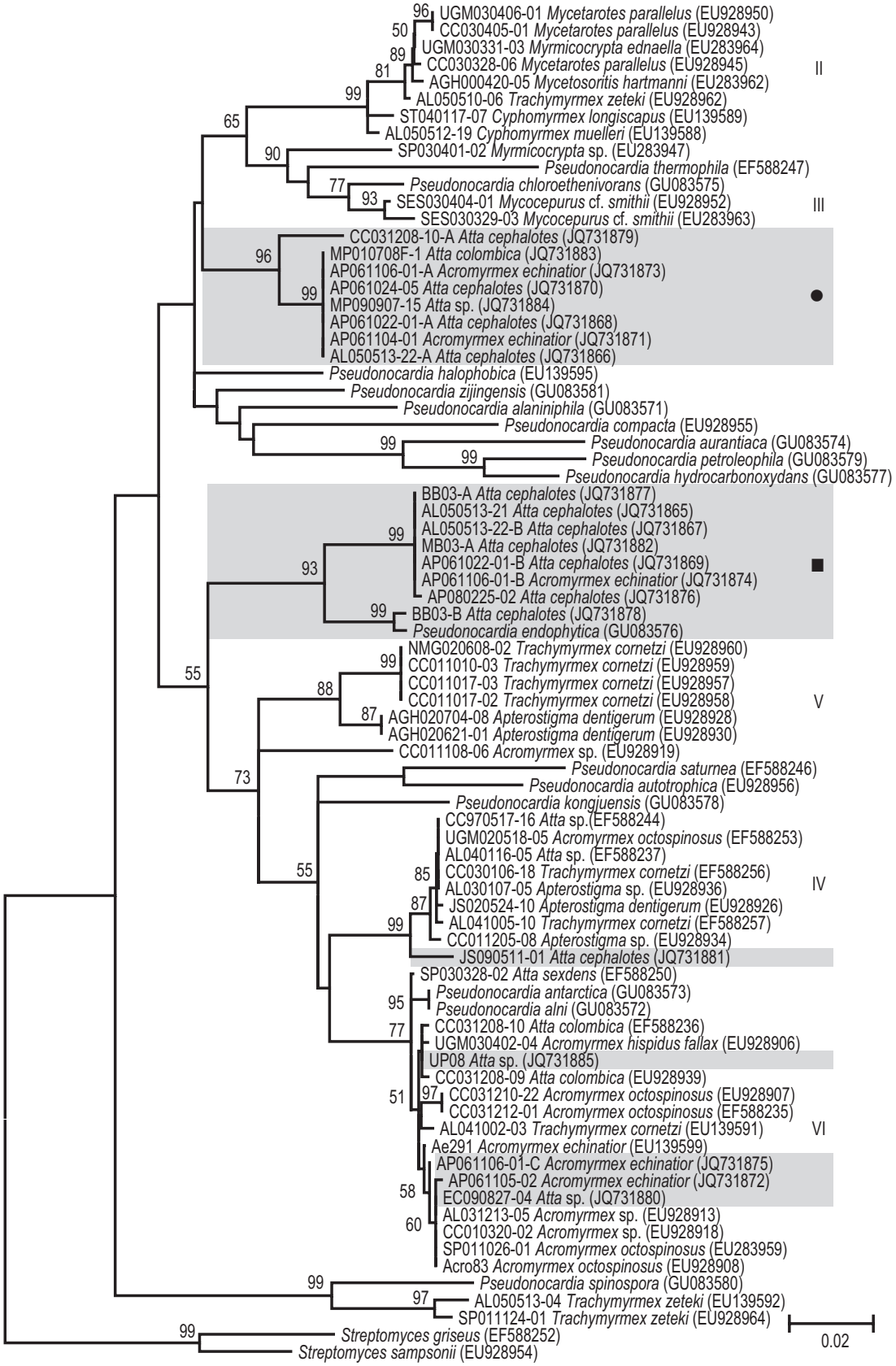

Figure 1. *Pseudonocardia* Phylogeny Elongation factor-Tu phylogeny of *Pseudonocardia* isolates from *Atta* and *Acromyrmex* generated in this study, along with other symbionts from across the diversity of fungus-growing ants. *Pseudonocardia* strains are labeled with the colony code and name of the ant from which they were isolated. Some *Atta*-associated strains of *Pseudonocardia* isolated in this study fall into clades with other leaf-cutting ant-associated *Pseudonocardia* (clades IV and VI, cf. Cafaro et al. 2011); however, this study also identified two previously undescribed clades of ant-associated *Pseudonocardia* (indicated with circle and square).

CHAPTER 3. ACQUISITION OF EXOSYMBIOTIC ACTINOBACTERIA BY *ACROMYRMEX* LEAF-CUTTER ANTS

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Co-authors contributed in the following ways: SEM designed experiments with input from other authors, performed all field work, experiments & data analysis and wrote the first draft. All authors edited and improved manuscript. MP provided useful guidance in experimental design. APT provided support in the field and experimental design advice. CRC provided lab materials and support.

I. ABSTRACT

Virtually all animals host beneficial microbial symbionts, some of which are critical for normal host development as well as for both organisms' survival. Leaf-cutter ants farm a fungal cultivar as their primary nutrient source. The ants also carry a specialized Actinobacterial exosymbiont, mainly in the genus *Pseudonocardia*, which produces antifungal compounds that inhibit *Escovopsis*, a specialized parasite on the ants' fungal garden. Major workers emerge from their pupal cases (eclose) symbiont-free, acquiring visible exosymbionts including *Pseudonocardia* 2-3 days post-eclosion. Here I use subcolony experiments, manipulating pupae, ants and fungus to investigate the mechanism of exosymbiont acquisition. I found successful transmission to all pupae fostered in subcolonies with major workers carrying Actinobacteria (n = 19). In contrast, no pupae from subcolony setups without major workers carrying Actinobacteria acquired visible exosymbiont coverage (n = 58). These results demonstrate that newly eclosed workers must interact with major workers carrying exosymbiont for successful acquisition to occur. I found that 6/11 ants exposed to majors 0-1 hours after eclosion acquired visible exosymbiont, while only 2/9 ants exposed 1-2 hours after eclosion acquired normally, suggesting a narrow exposure window for normal acquisition following which exosymbionts will not establish. This critical period in ant development associated with exosymbiont acquisition may help ensure specificity and partner fidelity. Together with studies of other symbioses, our work suggests that a critical

developmental period for symbiont acquisition may be a widespread mechanism hosts employ to limit cheating.

II. Introduction

Symbiosis, the living together of unlike organisms (de Bary 1879), has been and remains critically important to the evolution of life on Earth. The success of symbiosis depends on hosts' ability to properly acquire and maintain appropriate symbionts. Microbial symbionts are primarily either acquired from the environment (horizontal transmission), or vertically, from parent to offspring. Highly constrained transmission allows few opportunities for partner switching, encouraging increased partner fidelity, important for establishing the stable interactions important for developing symbioses (Sachs *et al*. 2011). This type of evolutionary constraint can be imposed through several mechanisms, including recognition through partner mediated molecular patterns by hosts as well as symbionts (Chaston & Goodrich-Blair 2010).

Leaf-cutting ants have been farming an obligate fungal cultivar mutualist for approximately 10 million (Schultz & Brady 2008). The ants rely on their fungal garden as a food source, in turn the cultivar depends on the ants to provide leaves as a nutrient source, to aid in combating microbial pests, and to propagate to new colonies (Hölldobler & Wilson 1990; Schultz & Brady 2008). Ants in the genus *Acromyrmex* host Actinobacteria that produce antimicrobial compounds capable of inhibiting *Escovopsis*, a specialized parasite on the ants' fungal cultivar. Actinobacteria include *Pseudonocardia*, shown in both bioassays and *in vivo* to produce compounds that inhibit *Escovopsis* growth (Currie *et al*. 1999, 2003; Poulsen *et al*. 2010; Cafaro *et al*. 2011). *Streptomyces* capable of producing antibiotic compounds have also been reported in

association with the ants, however their role remains unclear (Kost *et al*. 2007; Haeder *et al*. 2009; Barke *et al*. 2010; Schoenian *et al*. 2011). Within a nest only major workers carry visible Actinobacteria, while minor workers do not (Poulsen *et al*. 2002). These ants appear to provide the Actinobacteria with nutrition from glands and tubercles on the cuticle (Currie *et al*. 2006). *Atta* ants lack these structures and do not carry visible Actinobacteria (Currie *et al*. 2006). Mature leaf-cutting ant colonies produce tens to hundreds of alates, winged male and female reproductives. During mating flights hundreds of these virgin gynes and males leave their natal colonies, mating multiply before the queen ants return to earth to establish their own incipient colonies (Hölldobler & Wilson 1990). These queens are covered with a visible layer of exosymbiotic bacteria, primarily transmitted from vertically to offspring nests (Currie *et al*. 2006).

Acromyrmex octospinosus major workers acquire their bacterial exosymbiont in a both spatially and temporally characteristic pattern. Leaf-cutter ants require social facilitation to eclose properly (Hölldobler & Wilson 1990). These ants emerge from their pupal cases (eclose) symbiont-free and acquire the first visible traces of *Pseudonocardia* detectable by electron microscopy by two to three days after eclosion, and exosymbiont levels increase for 10-15 days (Poulsen, Bot, Currie, *et al*. 2003). Ants maintain a high level of symbiont coverage until they reach approximately 25 days post-eclosion, during the period when workers remain in the fungal garden, tending the cultivar (Poulsen, Bot, & Boomsma 2003). The decline in *Pseudonocardia* coverage coincides with the time the ants typically begin to leave the garden to forage (Poulsen, Bot, & Boomsma 2003). In contrast leaf-cutter ants in the genus *Atta* typically do not carry visible loads of *Pseudonocardia* (Currie *et al*. 2006) and metagenomics study have failed to find

any evidence of *Pseudonocardia* in the fungus garden of *Atta cephalotes* (Aylward *et al*. 2012). Metagenomic analysis of *Acromyrmex echinatior* and *Acromyrmex octospinosus* gardens found extremely low levels of Actinobacteria. If *Pseudonocardia* were present in these gardens, they were below the detection threshold of this technique (F. Aylward unpublished data).

The findings that major workers eclose Actinobacteria-free but obtain the first signs of growth after 2-3 days and low levels of Actinobacteria in fungus gardens, suggest that majors obtain Actinobacteria inocula from older bacteria-carrying workers and that this happens during a short window of time after eclosion. To explore whether acquisition is driven by the presence of Actinobacteria-carrying workers, I fostered newly eclosed *Acromyrmex* majors in the presence or absence of such older workers, as well as with *Atta* workers, not carrying the exosymbiont. Furthermore, I explored the time frame in which acquisition occurred by rearing newly eclosed majors in subcolonies where Actinobacteria-carrying majors where introduced at different time points. Our findings showed that Actinobacteria are transmitted during a short time window within colonies from older to younger major workers, and this mode of transmission implies long-term colony-level ant-Actinobacteria specificity. In order to test whether this is indeed the case, I isolated and sequenced DNA 16S rDNA of the Actinobacteria-symbiont *Pseudonocardia* from nine colonies, and compared these to sequence obtained when colonies were first brought back to the lab between five and nine years ago.

III. METHODS

ANT COLONIES

The exosymbiont source and stability experiments were performed using the following colonies maintained at the University of Wisconsin-Madison: *Acromyrmex echinatior*: AP061104-01 (P6) Costa Rica; AeP5 (P5) & CC031209-02, CC031212-01 (P8) Panama; EC110523-1 (CR1), EC110523-2 (CR2) & EC110523-3 (CR3) Costa Rica; *Acromyrmex hispidus fallax* UGM030327-02 Argentina, *Acromyrmex laticeps* CC030403-09 and UGM030330-04 Argentina; *Acromyrmex niger* CC030327-02 Argentina; *Acromyrmex octospinosus*: CC030403-09 (O8) Argentina; AL050505-11 (O13), CC031210-22 (O7), MP010908-1 (P11), SA010908-4, ST040116-01 (P10) & UGM020518-05 (P9) Panama; *Atta cephalotes*: AP061021-2 (G9) & AL050513-22 Costa Rica. Colonies were maintained at 24°C in the dark to mimic underground conditions, with overhead lights illuminated periodically for colony maintenance. Each ant colony was housed in a large outer plastic container (17.5 cm H x 29 cm W x 40 cm L) accommodating one smaller refuse dump container and one or two garden containers (ranging from 3.0 cm H by 7.5 cm L x 7.5 cm W to 11 cm H x 19.5 cm L x12.5 cm W, Pioneer Plastics). Each container had a 1 cm diameter hole drilled to allow ants to move in and out. Mineral oil was applied regularly to the top 4 cm of the outer container to prevent ants from escaping. Colonies were provisioned maple (*Acer* sp.) and oak (*Quercus* sp.) leaves (frozen in the winter and fresh during summer) three times per week. Leaves were supplemented with oatmeal, rice and cornmeal. Wet cotton balls were stored in the outer box to increase humidity.

Acquisition timing experiments were performed using *Acromyrmex octospinosus* colonies (G: SEM110828-1, M: RVC110826-1, R: RVC110813-3) collected in Heredia, Costa Rica in 2011. These colonies were maintained the in laboratory at the La Selva Biological station at 24°C

under ambient lab light conditions. Each colony was housed in a larger plastic container (28 cm H x 40 cm W x 56 cm L) accommodating one smaller plastic container for refuse dump and one to three smaller plastic containers (ranging from 3.0 cm H by 7.5 cm L x 7.5 cm W to 11 cm H x 19.5 cm L x12.5 cm W, Pioneer Plastics) enclosing the colony fungus gardens. These colonies were provisioned with local flora, including leaves and flowers from Needle Flower tree, *Posoqueria latifolia*, Tonka bean tree, *Dipteryx panamensis*, fig tree, *Ficus* sp., Sandbox tree, *Hura crepitans, Arrabidaea* sp., *Souroubea sympetala*, breadnut tree, *Brosimum alicastrum*, among others.

SUBCOLONY SET-UP: ACQUISITION SOURCE EXPERIMENT

I performed subcolony experiments to evaluate the components necessary for successful exosymbiont transmission. Subcolonies containers were small (4.0 cm H by 5.5 cm OD) clear plastic containers (Pioneer Plastics, Round Container 002C). After sterilizing the containers for at least 20 minutes using UV light, a Kimwipe moistened with distilled water was placed at the bottom to help provide humidity. A small (4.12 cm W x 4.12 cm L x 0.79 cm H) weigh boat (Fisher catalog #08-732-112) was placed on top of the Kimwipe, and then the fungus garden, ants and pupa were added. A ~1 cm^2 leaf fragments of pin oak (*Quercus palustris*) were added 24 hours or more after eclosion for the ants to cut and incorporate. I started by establishing that *Acromyrmex* ants could acquire normally in a subcolony set-up consisting of a focal pupa, 0.1 g fungus, 2 major workers and 4 minor workers (a ratio other authors have found to be stable (Abramowski *et al*. 2011), Figure 2, Table 1). I monitored subcolonies daily to record eclosion date and visible exosymbiont coverage level for the focal ant until she reached 14-21 days old;

defining acquisition as successful colonization leading to visible coverage. Colony components were replaced as necessary (dead nurse ants and fungal garden). I explored which of the following could act as a source of exosymbiont: 1) fungal garden; via cross-fostering with a related strain of fungus [a focal pupa, 0.1 g *Leucoagaricus* from *Atta cephalotes*, 2 major workers and 4 minor workers]; 2) conspecific worker ants; via cross-fostering with a related strain of ants with no visible Actinobacteria [a focal pupa, 0.1 g fungus garden, 2 major and 4 minor *Atta cephalotes* workers]; 3) minor workers* [a focal pupa, 0.1 g fungus garden, 8 minor workers]; 4) major workers* not carrying visible Actinobacteria [a focal pupa, 0.1 g fungus garden, 4 major workers cross-fostered with *Atta cephalotes* or fostered with minor workers only that had no visible Actinobacteria growth] and 5) major workers carrying visible Actinobacteria [a focal pupa, 0.1 g fungus garden, 4 major workers]; *note that caste is traditionally defined according to head size (Wilson 1983; Wetterer *et al*. 1998). In this case I roughly classified workers by size, then verified that all major ants carried visible exosymbiont, while minors did not. Also see Table 1 for an outline of experimental set-ups.

SUBCOLONY SET-UP: ACQUISITION TIMING EXPERIMENT

To determine whether there was a critical window for exosymbiont acquisition I started by cross-fostering *Acromyrmex* pupae with *Atta* workers. Once pupa eclosed I switched the *Atta* workers with *Acromyrmex* workers from the pupa's natal colony and allowed the ants to mature to 14-21 days old. No focal ants from these preliminary studies with switching between one and four days acquired exosymbiont. In parallel, our work showed that raising pupa fostered with minor workers alone lead to exosymbiont-free focal ants. Preliminary results suggested the critical

window was less than 24 hours long.

I defined the time of eclosion as the time when pupa could first begin to interact with the environment. Manipulating pupa to visually confirm verify the first holes in the pupal casevia light microscopy lead to extremely high mortality. However, I noted the pupa often began struggling to help free-themselves from their pupal membranes once this membrane was broken. I relied upon this coincident kicking-behavior to mark the time of eclosion. In addition, similar kicking behavior coincident with the membrane breaking has been previously reported in fire-ant eclosion (Lamon & Topoff 1985).

To determine the time after eclosion in which acquisition was most likely to predominantly occur, I fostered pupa with four minor workers with no visible exosymbiont from their natal colony. Subsequently, I added two majors with visible exosymbiont collected from the ants' natal garden at different time points after focal ant eclosion. Once these majors were added they remained in the subcolonies until the end of the experiment. I could not determine the age of these workers exactly, but select workers that likely had been involved in brood-care within the colony, and consequently likely were around the same age. Control colonies were set-up with these majors present initially, while majors were added to experimental subcolonies in the following time windows 0-1 (immediately on detection), 1-2, 3-4, 7-8 and 24-30 hours after eclosion. Leaf-fragments were added 24 hours after eclosion, to facilitate observing eclosion behavior.

LONG TERM STABILITY

Our findings from the first experiments show that Actinobacteria are transmitted during a short time window within colonies from older Actinobacter-acarrying major to newly eclosed majors. This mode of transfer implies long-term colony-level ant-Actinobacteria specificity, which implies that the Actinobacteria acquired at the time of the experiments should be genetically identical to when colonies were first brought to the lab. In order to test whether this was the case, I isolated and sequencing 16S rDNA of the Actinobacteria-symbiont *Pseudonocardia* from 9 colonies, and compared these to sequence obtained when colonies were first brought back to the lab between five and nine years ago. At the time of colony collection, bacteria had been isolated from individual *Acromyrmex* ants by scraping visible *Pseudonocardia* from their propleural plates onto chitin plates, allowing bacteria to grow for three weeks, then transferring colonies with Actinobacteria-like morphology (small, white dust balls) to yeast malt extract plates (see (Marsh *et al*. Submitted; Poulsen *et al*. 2005; Cafaro *et al*. 2011) for detailed descriptions of methods). I re-isolated Actinobacteria from colonies that had been maintained in the lab from five to nine years using the same methods. I compared 16S ribosomal RNA gene sequences to assess whether ant colonies maintained the same bacterial symbiont by comparison to the original isolates. Bacterial DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol described in (Marsh *et al*. Submitted; Poulsen *et al*. 2005; Cafaro *et al*. 2011) and genomic DNA was quantified by nanodrop photospectrometer (http://www.nanodrop.com) and diluted to 50 ng/μl using TE. I then amplified 16S rDNA using universal primers (27f 5'AGAGTTTGATCMTGGCTCAG'3 and 1492r 5'TACGGYTACCTTGTTACGACTT'3, (Giovannoni 1991)). Amplicons were sequenced on an ABI 3730xl DNA Analyzer at the University of Wisconsin Biotechnology Center (http://biotech.wisc.edu). I edited sequences

using Sequencher 4.5 (http://www.genecodes.com/), then ran a preliminary alignment using Clustal (http://www.clustal.org/). I edited the alignment using MacClade 4.07 (http://macclade.org/), then used Mega 5.0 (Tamura *et al*. 2011) to generate Maximum likelihood a phylogeny based on the Tamura-Nei model with all positions containing gaps and missing data eliminated.

IV. RESULTS

SOURCE OF EXOSYMBIONT TRANSMISSION TO NEWLY ECLOSED WORKERS

I explored the conditions under which newly eclosing *Acromyrmex* ants would acquire Actinobacteria exosymbionts. The majority of focal pupae in control subcolonies (containing focal pupa, fostering major and minor ants and leaf-fragments) acquired exosymbiont normally (*Ae* n = 5/6) (Table 1). The majority of pupae in subcolonies with colony components from the same natal colony acquired in the pattern reported by Poulsen and colleagues (Poulsen, Bot, Currie, *et al*. 2003).

I cross-fostered *Atta* and *Acromyrmex* pupae with majors and minors from their natal colonies, but with fungus garden fragments from ants of the other genera (e.g. *Atta* ants and pupae with *Acromyrmex* garden). Ants in these experiments suffered high mortality rates. No *Acromyrmex echinatior* ants lived five days post-eclosion so all results reported here are for *Acromyrmex octospinosus*. *Atta* ants did not acquire exosymbiont when raised with *Acromyrmex* associated fungus garden (with the potential to carry this bacterium) (*Atta* n = 5; raised on fungus from four different *Acromyrmex octospinosus* colonies). In contrast, *Acromyrmex* ants acquired normally when raised on *Atta* garden (*Ao* n = 7), suggesting that the garden is not a significant source of

exosymbiont for these ants. Together these results suggest that the fungal garden does not play a role in exosymbiont acquisition.

When *Acromyrmex octospinosus* (Ao) and *Acromyrmex echinatior* (*Ae*) pupae were cross-fostered with *Atta* garden and worker ants, *Acromyrmex* ants failed to acquire exosymbionts (*Ao* n = 32; *Ae* n = 5), suggesting that interaction with worker ants carrying Actinobacteria are key to exosymbiont acquisition. *Acromyrmex* ants raised with their own fungal garden, but *Atta* worker ants also did not acquire exosymbiont (*Ao* n = 14; *Ae* n = 5), confirming that this failure to acquire arose due to the lack of Actinobacteria-carrying workers.

Pupae raised with minor workers only failed to acquire Actinobacteria (*Ao* n = 7; *Ae* n = 4), while those raised with major workers (*Ao* n = 7; *Ae* n = 5) acquired bacteria. This suggests that majors are essential for successful exosymbiont transmission to occur. Since major workers carry visible exosymbiont on their exoskeletons while minor workers do not carry large quantities of Actinobateria, this suggests that the presence of workers carrying visible levels of exosymbiont is necessary for acquisition. To further investigate this, I fostered pupae with major workers carrying no visible exosymbiont, and none of these pupae acquired exosymbionts (*Ao* n = 7; *Ae* n =3). These results suggest that direct contact with major workers carrying visible exosymbiont is necessary in order for successful exosymbiont transmission to occur (Figure 2).

ACQUISITION TIMING

Mortality was high across all fostered pupae (control 10/19, 0-1 hours 11/22, 1-2 hours 8/20, 3-4 hours 10/10, 7-8 hours 9/18 and 24-30 hours 11/15), however a clear trend in acquisition timing

emerged (Table 2, Figure 3). All surviving control ants acquired exosymbiont normally, as did 55% of ants in the 0 to 1 hour window, and 20% of the ants in the 1 to 2 hour window. No ants acquired exosymbionts when exposed to major workers with visible exosymbiont outside this narrow timeframe.

SYMBIONT STABILITY

Pseudonocardia isolated from the workers from the same parent colonies 1 months to 9 years apart appear to have retained the same symbiont as supported by 99-100% 16s rDNA sequence similarity over time. In addition, all symbionts isolated from a given colony group within the same phylogenetic clade (Figure 4). While some sequence differences were observed, none occurred at greater levels than might be accounted for by sequencing error suggesting that symbionts have remained stable within these colonies since collection.

V. DISCUSSION

I experimentally explored the conditions required for *Acromyrmex* leaf-cutters to properly acquire their actinobacterial exosymbiont. First I confirmed that newly eclosed workers could acquire their exosymbiont in small subcolonies with leaf fragments, fungal garden, and adult major and minor workers present. Second, switching experiments eliminate that fungal-garden and minor workers as a source for transmission of this bacterium. This shows that the presence of Actinobacteria-carrying major workers is necessary for successful transmission to occur. I conclude that major worker to major worker transmission is the sole route used in this system.

I used the information gleaned from the experiments described above to explore host influence over exosymbiont establishment through imposition of a critical acquisition period. I did this by manipulating the timeframe during which callow workers were exposed to major workers with Actinobacteria cover necessary for successful acquisition. This resulted in a sharp acquisition curve, with all control ants acquiring exosymbiont, the majority of the ants acquiring after short time frames (0-1 hours post-eclosion), dropping quickly with time so that no ants exposed to exosymbiont after more than three hours post-eclosion acquired Actinobacteria. This narrow window of a few hours during which the ant could normally acquire exosymbionts would increase host-symbiont specificity by limiting the period during which the ants could be colonized by bacteria and possibly limiting the number of exosymbiont strains establishing. Observations during this critical period revealed that major workers interact closely with newly eclosed, antennating, walking on and even carrying the callow around the subcolony. While no behavior that seemed as an obvious mechanism for transmission of the exosymbiont was observed, it is possible that general interactions are sufficient or further work might be able to additionally narrow the interactions required for successful colonization. Work in other symbioses suggests that critical windows for symbiont acquisition maybe widespread. For example, researchers recently experimentally defined a critical developmental window for acquisition of a horizontally transmitted *Burkholderia* midgut symbiont by the alydid stinkbug, *Riptortus pedestris* (Kikuchi *et al*. 2011). In addition, work has shown the importance of this timing during succession in the beneficial symbionts in the gut community of the European medical leech (*Hirudo verbana*) (Rio *et al*. 2009). A limited symbiont acquisition periods may

be a common feature of symbiotic interactions, perhaps as a mechanism to increase colonization specificity.

Sequences from *Pseudonocardia* exosymbiotic bacteria isolated from nine *Acromyrmex* colonies remained highly similar from original colony collection to the present, suggesting that the same bacterial species had been stably associated with these colonies for up to nine years in the lab. Our culture-dependent findings concur with Andersen and colleagues (Andersen 2011) who used culture-independent pyrosequencing to examine the bacteria associated with the propleural plates of *Acromyrmex echinatior*. Similar to this work and other studies with *Acromyrmex* (Poulsen *et al*. 2005; Cafaro *et al*. 2011), Andersen found *Pseudonocardia* mapping to one of two clades. These authors also examined a time-course of samples, concluding that the exosymbiont appeared to be stable over the life of the colonies examined. However, cross-fostering studies have demonstrated that *Pseudonocardia* can switch between members of these clades (Armitage *et al*. 2011; Marsh unpublished data). However, more work will be necessary to fully explain the evolutionary mechanisms behind larger patterns of diffuse coevolution between leaf-cutter ants and their exosymbiont *Pseudonocardia*.

The elements of this study highlight the importance of ant determinants of exosymbiont species-specificity in establishment and maintenance of the leaf-cutter ant symbiosis. These findings imply possible mechanisms limiting exosymbiont transmission within the symbiosis including specific interaction and timing. They also provide support for long-term stability (over years) of *Pseudonocardia* within the system. Proper symbiont establishment and maintenance are

essential phases in initiating stable symbiotic interactions. Similar mechanisms to maintain specificity are likely to occur in acquisition and maintenance phases within other symbioses.

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VII. References

Abramowski D, Currie CR, Poulsen M (2011) Caste specialization in behavioral defenses against fungus garden parasites in Acromyrmex octospinosus leaf-cutting ants. *Insectes Sociaux*, **58**, 65–75.

Andersen SB (2011) Dynamics of ant-microbial interactions: Coevolution along the parasitism-mutualism continuum.

Armitage SAO, Broch JF, Fernandez Marin H, Nash DR, Boomsma JJ (2011) Immune Defense in Leaf-Cutting Ants: a Cross-Fostering Approach. *Evolution*, **65**, 1791–1799.

Aylward FO, Burnum KE, Scott JJ, *et al*. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus garden. *ISME J*.

Barke J, Seipke RF, Grüschow S, *et al*. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant Acromyrmex octospinosus. *BMC Biology*, **8**, 109.

de Bary HA (1879) *Die Erscheinung der Symbiose*. Karl J. Trubner, Strasburg.

Cafaro MJ, Poulsen M, Little AEF, *et al*. (2011) Specificity in the symbiotic association between fungus-growing ants and protective Pseudonocardia bacteria. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 1814–1822.

Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*, **34**, 41–58.

Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos*, **101**, 91–102.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science*, **311**, 81–83.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.

Giovannoni SJ (1991) The polymerase chain reaction. In: *Nucleic Acid Techniques in Bacterial Systematics* (eds Strackebrandt E, Goodfellow M), pp. 115–175. John Wiley & Sons, Chichester.

Haeder S, Wirth R, Herz H, Spiteller D (2009) From the Cover: Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis. *Proceedings of the National Academy of Sciences*, **106**, 4742–4746.

Hölldobler B, Wilson EO (1990) *The Ants*. Belknap Press of Harvard, Cambridge, MA.

Kikuchi Y, Hosokawa T, Fukatsu T (2011) Specific Developmental Window for Establishment of an Insect-Microbe Gut Symbiosis. *Applied and Environmental Microbiology*, **77**, 4075–4081.

Kost C, Lakatos T, Böttcher I, *et al*. (2007) Non-specific association between filamentous bacteria and fungus-growing ants. *Naturwissenschaften*, **94**, 821–828.

Lamon B, Topoff H (1985) Social Facilitation of Eclosion in the Fire Ant, Solenopsis-Invicta. *Developmental psychobiology*, **18**, 367–374.

Marsh SE, Poulsen M, Gorosito NB, *et al*. (Submitted) Optimization of isolation methods suggests the presence of an association between Pseudonocardia symbionts and Atta leaf-cutting ants. *Symbiosis*.

Poulsen M, Bot ANM, Boomsma JJ (2003) The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. *Naturwissenschaften*, **90**, 406–409.

Poulsen M, Bot ANM, Currie CR, Boomsma JJ (2002) Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in Acromyrmex leaf-cutting ants. *Insectes Sociaux*, **49**, 15–19.

Poulsen M, Bot ANM, Currie CR, Nielsen MG, Boomsma JJ (2003) Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant Acromyrmex octospinosus. *Functional Ecology*, **17**, 260–269.

Poulsen M, Cafaro M, Boomsma JJ, Currie CR (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of Acromyrmex leaf-cutting ants. *Molecular ecology*, **14**, 3597–3604.

Poulsen M, Cafaro MJ, Erhardt DP, *et al*. (2010) Variation in Pseudonocardia antibiotic defence helps govern parasite-induced morbidity in Acromyrmex leaf-cutting ants. *Environmental Microbiology Reports*, **2**, 534–540.

Rio RVM, Maltz M, McCormick B, Reiss A, Graf J (2009) Symbiont Succession during Embryonic Development of the European Medicinal Leech, Hirudo verbana. *Applied and Environmental Microbiology*, **75**, 6890–6895.

Sachs JL, Essenberg CJ, Turcotte MM (2011) New paradigms for the evolution of beneficial infections. *Trends in Ecology & Evolution*, **26**, 202–209.

Schoenian I, Spiteller M, Ghaste M, *et al*. (2011) Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 1955–1960.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5435–5440.

Tamura K, Peterson D, Peterson N, *et al*. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, **28**, 2731–2739.

Wetterer JK, Gruner DS, Lopez JE (1998) Foraging and nesting ecology of Acromyrmex octospinosus (Hymenoptera : Formicidae) in a costa Rican tropical dry forest. *Florida Entomologist*, **81**, 61–67.

Wilson EO (1983) Caste and Division of Labor in Leaf-Cutter Ants (Hymenoptera, Formicidae, Atta) .4. Colony Ontogeny of Atta-Cephalotes. *Behavioral Ecology and Sociobiology*, **14**, 55–60.

VIII. TABLES (begin on next page)

Table 1. Exosymbiont Source experiment. The first column outlines each experiment, with subsequence columns providing the components in each subcolony: species of pupae (focal ant) and adult ants ; number of exosymbiotic and aposymbiotic majors, and minors; fungus source colony ant species, and finally results (mortality and proportion of ants acquiring).

Experiment	Pupae species	Adult species	# Majors		# minors	Fungus source	Survival to eclosion	# acquired exosymbiont
			Exosymbiotic	Aposymbiotic				
Subcolony function	*Acromyrmex echinatior*	*Acromyrmex echinatior*	2	0	4	*Acromyrmex echinatior*	6/10	5/6
Fungus garden & ant switch	*Acromyrmex echinatior*	*Atta cephalotes*	-	2	4	*Atta cephalotes*	5/6	0/5
	Acromyrmex octospinosus	*Atta cephalotes*	-	2	4	*Atta cephalotes*	32/61	0/32
Fungus garden switch	*Acromyrmex echinatior*	*Acromyrmex echinatior*	2	-	4	*Atta cephalotes*	0/4	-
	Acromyrmex octospinosus	*Acromyrmex octospinosus*	2	-	4	*Atta cephalotes*	7/12	0/7
	Atta cephalotes	*Atta cephalotes*	-	2	4	*Acromyrmex octospinosus*	5/6	0/5
***Atta* ant cross-foster**	*Acromyrmex echinatior*	*Atta cephalotes*	-	2	4	*Acromyrmex echinatior*	5/6	0/5
	Acromyrmex octospinosus	*Atta cephalotes*	-	2	4	*Acromyrmex octospinosus*	14/20	0/14
minor workers	*Acromyrmex echinatior*	*Acromyrmex echinatior*	-	-	8	*Acromyrmex echinatior*	4/6	0/4
	Acromyrmex octospinosus	*Acromyrmex octospinosus*	-	-	8	*Acromyrmex octospinosus*	7/9	0/7
Major workers	*Acromyrmex echinatior*	*Acromyrmex echinatior*	4	-	-	*Acromyrmex echinatior*	5/8	5/5
	Acromyrmex octospinosus	*Acromyrmex octospinosus*	4	-	-	*Acromyrmex octospinosus*	7/9	7/7
Major workers without exosymbiont	*Acromyrmex echinatior*	*Acromyrmex echinatior*	-	2	4	*Acromyrmex echinatior*	3/3	0/3
	Acromyrmex octospinosus	*Acromyrmex octospinosus*	-	2	4	*Acromyrmex octospinosus*	7/9	0/7

Table 2. Acquisition timing. The accumulated number of ants from each of three colonies acquiring exosymbiont or remaining visibly clear of Actinobacteria (clean). Also including mortality statistics for ants dying before 14 days or exosymbionts became visible (not included in total). * While only four ants lived from the original set of three colonies, 7 additional sub-colonies were set-up in the lab for follow-up. All lived to 14 days, none acquired visible exosymbiont.

	Acquired		Clean		Number		
	#	%	#	%	Living	Dead	Total
Control	9	100%	0	0%	9	10	19
0-1 hours	6	55%	5	45%	11	11	22
1-2 hours	2	22%	7	78%	9	8	17
3-4 hours	0	NA	0	NA	0	10	10
7-8 hours	0	0%	3	100%	3	9	12
24-30 hours	0	0%	4	100%	4*(11)	11	15*(22)

IX. Figures

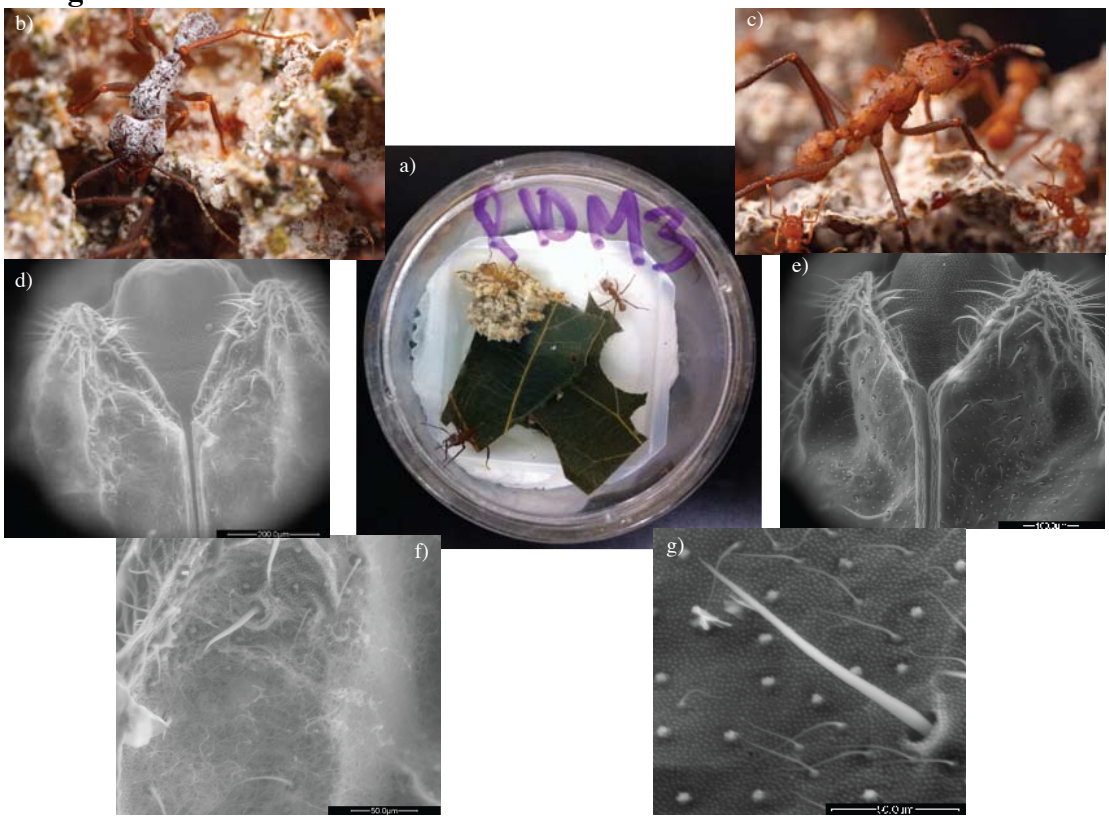

Figure 1. Experimental subcolony setup along with symbiotic and aposymbiotic ants. a) A subcolony with newly eclosed worker (light ant) and two major workers (darker ants), fungus garden and leaf fragments; photographs contrasting b) an adult symbiotic ant (b) and an adult aposymbiotic ant (c); environmental scanning electron micrographs contrasting a 21 day old a symbiotic ant–note visible Actinobacteria (d) larger field &(f) closer view, with a 21 day old aposymbiotic ant carrying no visible Actinobacteria (e) larger field and (g) closer view. (b and c photos by Alex Wild, other photos by Sarah Marsh)

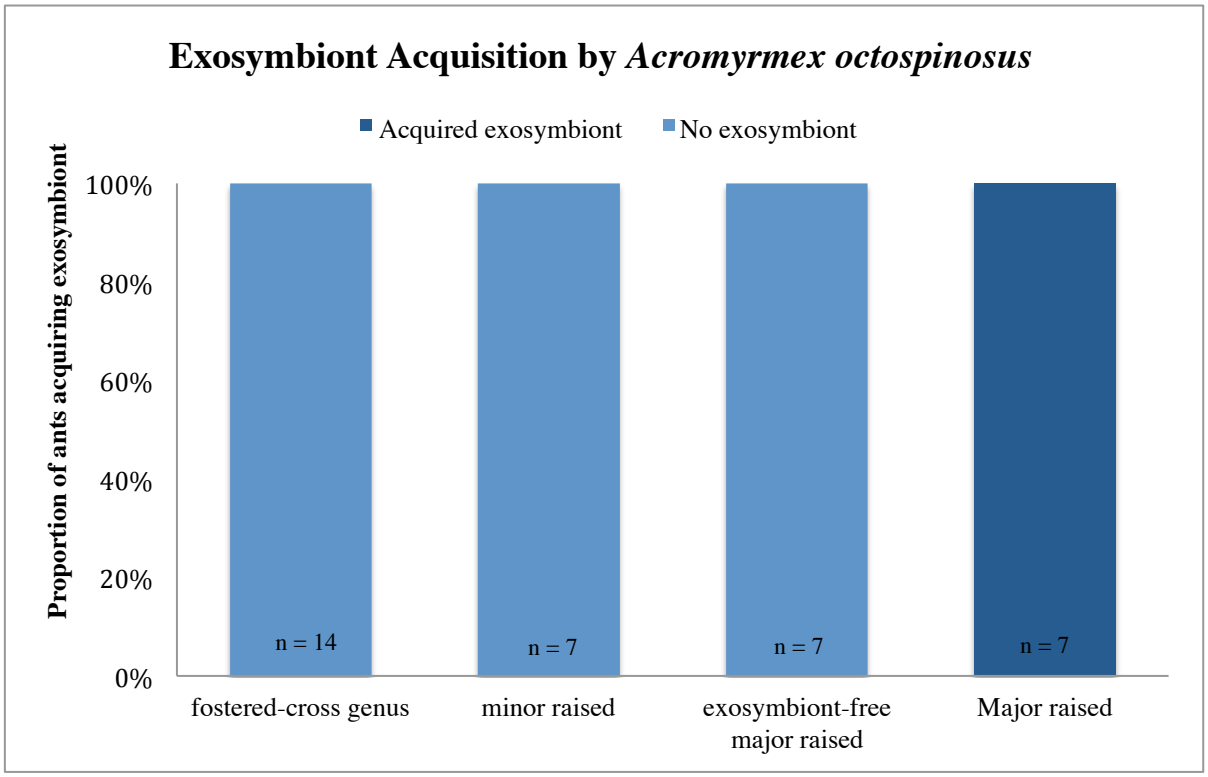

Figure 2. Conspecifics necessary for *Acromyrmex* to successfully acquire exosymbiont. *Acromyrmex octospinosus* successfully acquired their exosymbiotic bacteria only when pupae were raised in the presence of major workers carrying exosymbiont themselves. *Acromyrmex echinatior* ants show the same acquisition pattern (total n = 16; data not shown, **Table 1**).

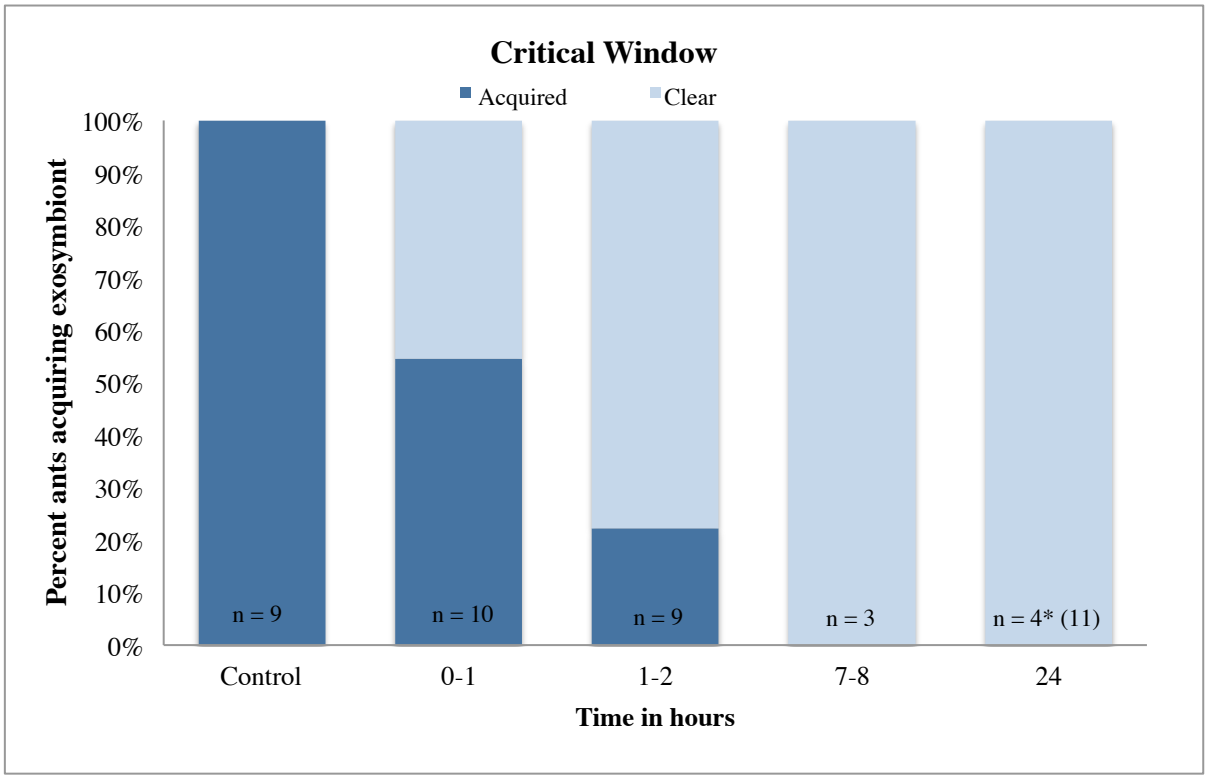

Figure 3. Critical Window Period for exosymbiont acquisition in *Acromyrmex octospinosus*. The chart shows the percentage of ants acquiring visible exosymbiont after being exposed to major workers carrying exosymbiont at varying times after eclosion (control subcolonies contained majors from the time of set-up).

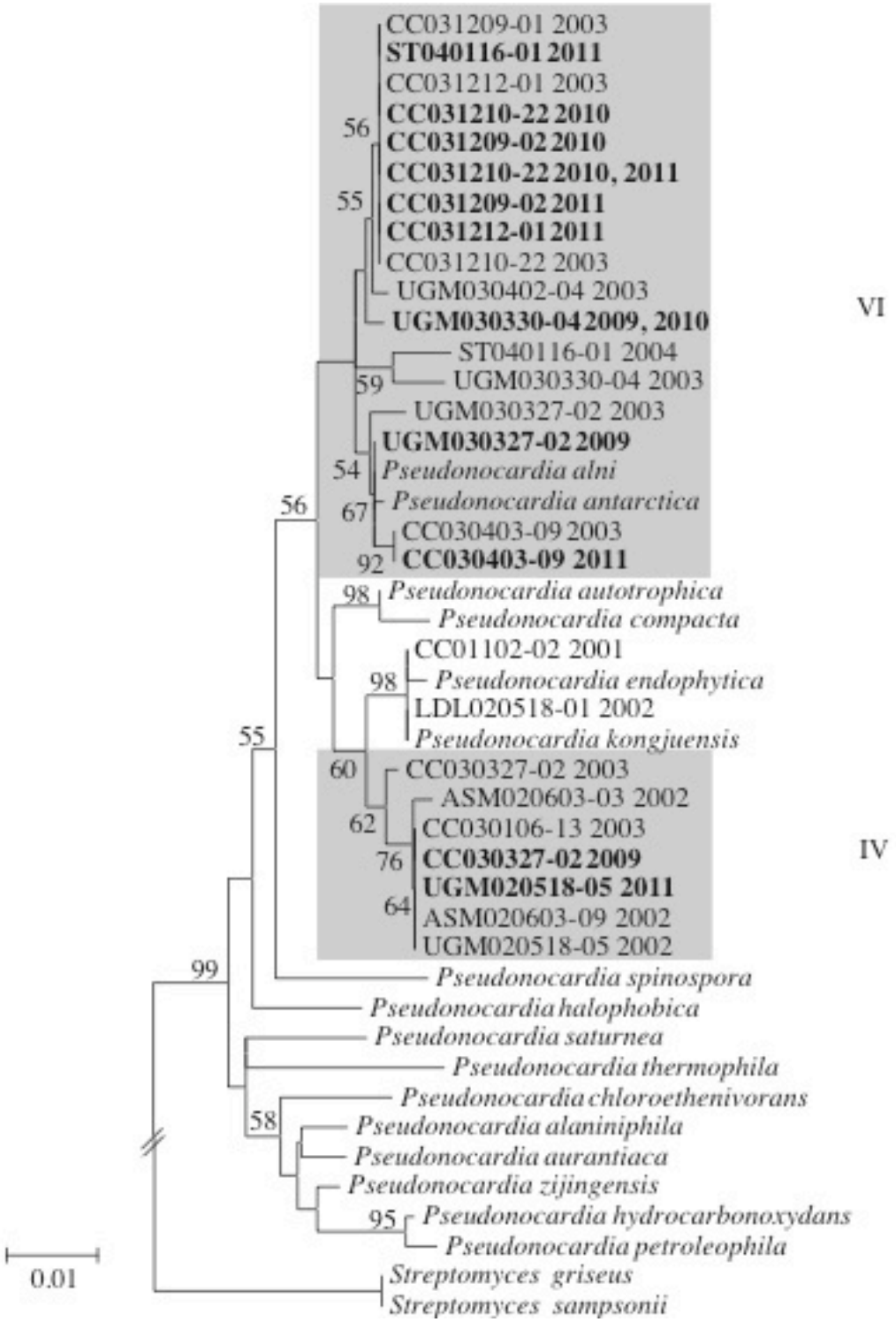

Figure 4. Phylogenetic tree showing bacterial sequences associated with individual leaf-cutter ants colonies over time. The phylogenetic tree above was created from 16s rDNA sequences of *Pseudonocardia* exosymbionts obtained from leaf-cutter ants at the time of colony collection (regular text) and five to nine years later (**bold text**). Note that the bacteria isolated from all colonies remains in the same clade over this time-scale.

CHAPTER 4. GENE EXPRESSION DIFFERENCES BETWEEN APOSYMBIOTIC AND SYMBIOTIC LEAF-CUTTER ANTS (*ACROMYRMEX ECHINATIOR*)

Manuscript in preparation: Sarah E. Marsh, Amy Cavanaugh & Cameron R. Currie

Co-authors contributed in the following ways: SEM designed experiments with input from other authors, SEM and AC performed experiments; SEM analyzed data and wrote the first draft of the manuscript. CRC provided lab materials and support.

I. ABSTRACT

Symbiont maintenance is a critical, yet little understood, stage in symbiotic interactions. Leaf-cutter ants' establishment of their visible exosymbiont (genus *Pseudonocardia*) provides a useful window to explore this phenomenon. The ants' bacterial exosymbiont produces antifungal compounds that inhibit *Escovopsis*, a specialized parasite on the ants' obligate fungal garden symbiont. In exchange for this antibiosis, the ants appear to provide their exosymbiont with nutrition. Here, I investigate the dynamics of this ant-bacterial symbiosis by examining gene expression changes in *Acromyrmex echinatior* ants with and without visible exosymbionts. I found 441 significant changes in gene expression in aposymbiotic ants, corresponding to 78 downregulated and 363 upregulated genes, including two chitinases. Chitins have been broadly implicated in symbiosis as Host Associated Molecular Patterns (HAMPs), perhaps unnecessary in aposymbiotic ants. Only three Gene Ontology (GO) categories showed significant changes in differential expression at the $p = 0.05$ level after Bonferroni correction for multiple testing, two cellular component terms—GO:0016021 integral to membrane and GO:0005576 extracellular region (corresponding to the location of the symbiont) and one Biological Process term—GO:0055114 oxidation-reduction processes (corresponding to differential respiration rates between aposymbiotic and symbiotic ants). I found upregulation was detected in the NF-KappaB pathway in aposymbiotic ants, suggesting that the host may be tuning down immune function, in particular microbial response, in response to the symbiosis, hence making them more susceptible

to infection. This difference may result in reads from fewer fungal garden and bacteria of all types detected in aposymbiotic ants. In addition, in symbiotic ants, *Pseudonocardia* were the most abundant bacteria detected (4.3% of total reads mapping to any genome), while other potential exosymbiotic bacteria made up less than 0.1% of reads. This difference indicates that *Pseudonocardia* makes up the majority of the exosymbiont population in 10-day-old ants. Future exploration of this system could provide useful insights into initial differences in expression over the developmental course of the symbiosis.

II. INTRODUCTION

Initiation is a critical period in beneficial symbioses. If host-symbiont interactions do not proceed smoothly at this stage, the mutualism may be in danger of breaking down (reviewed in Sachs & Simms 2006). Researchers have begun to explore the molecular-genetic underpinnings of colonization in other beneficial symbiotic systems. The most well understood is the Hawaiian bobtail squid (*Euprymna*)-*Vibrio* symbiosis, however other systems have been and are being examined (reviewed in Chaston & Goodrich-Blair 2010). This work has revealed that chitins, commonplace constituents of the exterior of many eukaryotes including invertebrates, and lectins are frequently used as Host Associated Molecular Patterns (MAMPs). In the *Euprymna*-*Vibrio* symbiosis, the host produces a mucus which appears to non-specifically bind many species of bacteria, then selects for *Vibrios* as they travel to pores within the light organ (Nyholm 2000). An alternative mechanism may be at work within other symbioses in which high binding specificity acts initially to limit the interactions to the species or even strain of interest (reviewed in Chaston & Goodrich-Blair 2010). Additional interest has been focused on how the host's immune system recognizes and reacts to beneficial microbes. In particular researchers have examined the immunity include the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KappaB)

pathway, Mitogen Activated Protein-Kinase (MAPK) pathway and Kruppel-like regulators (e.g. Chun *et al.* 2008).

Researchers have been studying the leaf-cutter ant symbiosis since the 1800's (e.g. Belt 1874). In this obligate mutualism, the ants farm a fungal cultivar as their primary food source. To help maintain their fungal garden the ants also associate with an exosymbiont in the genus *Pseudonocardia* that produces antifungal compounds targeting *Escovopsis*, a specialized parasite on the cultivar. As hosts, the ants appear to provide this beneficial bacterium with nutritional support. The ants eclose from their pupal membranes symbiont-free, acquiring visible *Pseudonocardia* loads within days. By 10 to 15 days they ants *Pseudonocardia* loads typically peak, waning again around day 25 when they begin to leave the nest to forage (Poulsen *et al*. 2003). The molecular basis of neither colonization nor maintenance has yet been explored in this system.

Perturbations of colonization can presage disease states, thus understanding colonization is vitally important to improving our knowledge of health and disease states themselves. Here, I explore the molecular mechanisms involved in and consequences of carrying an exosymbiont in *Acromyrmex* leaf-cutter ants. I generate exosymbiont free ants and compare global gene expression in these ants with age-matched symbiont-carrying counterparts. I examine overall changes in gene expression for patterns in Gene Ontology terms (GO), as well as examining expression changes in genes and pathways previously implicated in symbiotic interactions.

III. METHODS

LEAF-CUTTER ANT COLONIES

RNAseq experiments were done using a single colony of *Acromyrmex echinatior* leaf-cutter ants

collected in Gamboa, Panama in 2008. After collection, the colony (MP011108-2) has been maintained in the Currie-lab at the University of Wisconsin-Madison at 24°C in the dark to mimic underground conditions, with overhead lights only illuminated periodically. The colony is housed in a set of plastic containers; a large outer container (28cm H x 40cm W x 56cm L) accommodating one smaller plastic container for refuse material (dump) and two smaller plastic containers (ranging from 3.0 cm high by 7.5 cm x 7.5 cm to 11 cm high by 19.5 cm long by 12.5 cm wide) enclosing the fungus gardens. Each inner container had a 1 cm diameter hole drilled to allow ants to move in and out. Mineral oil was regularly applied to the top 4 cm of each outer container to prevent the ants from escaping. The ants are provisioned with maple and oak leaves (frozen in the winter months and fresh during summer) three times per week. Leaves are supplemented with oatmeal, rice and cornmeal. Wet cotton balls are periodically added to the outer box to increase humidity, as needed.

Ants used in this experiment were housed in small subcolonies. Each subcolony contained one focal pupa, two major worker ants with visible *Pseudonocardia*, four minor workers, and 0.1 g of fungal garden, a stable ratio (Abramowski *et al*. 2011) (Figure 1). Subcolonies were maintained in small clear plastic containers (4.0cm H by 5.5cm OD, Pioneer Plastics, Round Container 002C). Prior to use, each container was sterilized for at least 20 minutes using ultraviolet light, and a Kimwipe moistened with distilled water was placed at the bottom to help control humidity. A small (4.12cm W x 4.12cm L x 0.79cm H) weigh boat (Fisher catalog #08-732-112) was placed on top of the Kimwipe, and then the fungus garden, ants and pupa were added along with a ~1 cm^2 leaf fragments of pin oak (*Quercus palustris*) for the ants to cut and incorporate. Aposymbiotic ants were generated by first creating a generation of

Pseudonocardia-free major workers through rearing with minor workers alone (see Chapter 3 for details), then using these *Pseudonocardia*-free ants majors in place of majors carrying exosymbiont. Newly eclosed ants were monitored and collected for immediate RNA extraction at 10 days old between 9 am and 11 am on the morning to control for possible circadian effects.

RNA ISOLATION AND SEQUENCING

RNA was isolated using a modified version of the Masterpure Epicentre Complete RNA Purification kit (catalog# MC85200) with an additional 30 minute/37C DNase incubation step (RNase-Free DNase I, Epicentre Catalog #D9902K). Quality of extracted total RNA was verified by Bioanalysis. 0.5 μg total RNA from each of four aposymbiotic and four symbiotic ants from four ants was combined into 2 pools. Pooled samples were then polyA selected and tagged (ATCACG, GATCAG) according to the Illumina TruSeq Sample Preparation v2 guide protocol (Part# 15026495) with Illumina reagents at the University of Wisconsin's Biotechnology Center's Gene Expression Center. Samples were sequenced in one lane of a 100 bp single-end run, resulting in 9.73 Gb of reads (see Table 1 for details).

COMPUTATIONAL ANALSIS

I used the SolexaQA suite for quality assessment and trimming (Cox *et al*. 2010). I used SolexaQA to assess initial run quality, specifying a range of p-values 0.001, 0.01, 0.05 and 0.1. I then ran DynamicTrim, which finds the longest run of bases called with confidence above a specified level as above. Next I ran LengthSort to eliminate all reads under 75, 50 and 25 bp. To determine parameters best above to maximize quality control without losing a large amount of information, I mapped the reads to the existing *Acromyrmex echinatior* genome, assembly 2.0 using the tuxedo package described below. Based on this iteration through the parameter space

to find the highest mapping values with the greatest number of reads, I used a cutoff of p = 0.01, including all reads more than 25 bp long for all additional work.

I ran the Tuxedo suite of programs (using Tophat to call Bowtie, Cufflinks to examine differential expression, finally visualizing the analysis with CummeRbund) following a recent Nature Protocol (Langmead *et al*. 2009; Trapnell *et al*. 2009, 2012) to map the reads to the *Acromyrmex echinatior* draft genome assembly version 2.0, official gene set version 3.8 and examine differential expression. I ran Tophat, calling Bowtie with default setting to map reads to the *Leucoagaricus* draft genome (Currie & Aylward unpublished data). I ran Bowtie (settings: -a --best --strata -v 2 -m 3) to map reads back to microbial genomes including five strains of garden bacteria, *Enterobacter aerogenes* sp. FGI35, *Klebsiella variicola* sp. At-22, *Pantoea* sp. At-9b, *Pseudomonas plecoglossicida* sp. FGI182, and *Serratia* sp. FGI94 (Aylward *et al*. 2012). I also ran Bowtie to map the reads against a composite of two *Streptomyces* gnomes, increasing the allowed number of matches to 6 (*Streptomyces griseus* subsp. *griseus* NBRC 13350 (Ohnishi *et al*. 2008) and *Streptomyces coelicolor* A3(2) (Hsiao & Kirby 2007)), four finished *Wolbachia* genomes increasing the allowed number of matches to 12 (*Wolbachia* strain TRS, human filarial parasitic nematode *Brugia malay*i (Foster *et al*. 2005); *Wolbachia pipientis* strain *w*Pip, *Culex pipiens* (Klasson *et al*. 2008); *Wolbachia pipientis* strain *w*Mel, *Drosophila melanogaster* (Wu *et al*. 2004); and *Wolbachia pipientis* strain *w*Ri, *Drosophila simulans* (Klasson *et al*. 2009), a composite of six *Mycoplasma* genomes, increasing the allowed number of matches to 18 (*Mycoplasma capricoluma* ATCC 27343 uid58525, *Mycoplasma gallisepticuma* R low uid57993 (Papazisi 2003), *Mycoplasma genitalium* G37 uid57707 (Fraser *et al*. 1995), *Mycoplasma hyopneumoniae* 232 uid58205 (Minion *et al*. 2004), *Mycoplasma hyorhinis* HUB 1 uid51695

(Liu *et al*. 2010), *Mycoplasma mycoides* SC PG1 uid58031(Westberg 2004)) and against a composite of ten *Pseudonocardia* draft genomes increasing the allowed number of mismatches to 30 (*Pseudonocardia dioxanivorans* sp. CB1190, free-living (Mahendra 2005)*; Pseudonocardia* sp. P1 (*Acromyrmex octospinosus*) and *Pseudonocardia* sp. P2 (*Acromyrmex octospinosus*) (Barke *et al*. 2010); *Pseudonocardia saturnea* sp. NRRL B-16172*, Pseudonocardia* sp. AL050513-04 (*Tracymyrmex zeteki*), *Pseudonocardia* sp. CC030328-06 (*Mycerotes parallelus*), *Pseudonocardia* sp. CC011120-04 (*Apterostigma dentigerum*), *Pseudonocardia* sp. CC031212-01 (*Acromyrmex echinatior*), *Pseudonocardia* sp. CC031210-22, (*Acromyrmex octospinosus*), and *Pseudonocardia* sp. AL041002-03 (*Tracymyrmex cornetzi*) (Suen, Klassen & Currie unpublished data). These bacteria were selected due to affiliation with the system and based on common results when a subset of the data was BLASTned against the non-redundant database (Altschul 1997).

To assign Gene Ontology (GO) terms, I ran the published *Acromyrmex echinatior* protein file OGS v3.8 through the InterPro pipeline (Hunter *et al*. 2011), then consolidated GO terms by category (biological process, cellular component, and molecular function). I leveraged the differential expression results from the Tuxedo suite described above to run Goseq, a program designed to find significant changes in expression by GO term (Young *et al*. 2010). This package is designed to assess and correct for differences in mRNA length, which can bias expression analyses. I also ran the publically available protein file through the KEGG Automated Annotation Server (KAAS) (Moriya *et al*. 2007). I used this KAAS annotation to interrogate genetic pathways of interest *in silico*. This included genes previously implicated in symbiosis and those involved in immune response (JAK-STAT, MAPK, NF-KappaB, TOLL-

like, Chemokine signaling and Kruppel-like regulators) as well as Circadian rhythms (mammalian & fly models) as a control. I then combined this automated annotation with differential expression results to examine possible changes in pathways of interest.

IV. RESULTS

The RNAseq run yielded 49.7 and 46.6 million reads from aposymbiotic and symbiotic ants, respectively (Table 1). The heatmap of read quality indicated that while part of the flow cell appeared to fail (grey and black squares), read quality was generally high (light colored squares) (Figure 2). Note the similarity of the heatmaps, graphs and histograms between the two samples—indicating that each set of tags experienced similar sequencing conditions. In both cases, the heat maps show that seven tiles (2201, 2202, 2204-2208) failed completely, generating no reads that passed Illumina's initial data processing pipeline's quality threshold. This was likely due to air bubbles or other loading errors. Plotting read position against the likelihood of an error revealed that while the mean probability of an error in any position along the read did increase slightly along the read, these values were also generally low (Figure 3a, 3b). I generated histograms to examine the longest segment of high quality bases within each read using dynamic trimming. This resulted in a generally bimodal distribution with the majority of the reads retaining their full length (100 bp), with a second group of reads 34 base pairs long due to the errors discussed above (Figure 3c, 3d). Sequencing results were similar for both tags across comparisons, and neither exhibited detectable bias at the sequencing level.

I optimized read cleanup, balancing high read quality with maintaining high read number, (parameters: $p = 0.01$, minimum read length = 25 bp). Next I mapped the trimmed reads to the

Acromyrmex echinatior ant genome, *Leucoagaricus* fungal cultivar genome and a variety of bacterial genomes associated with the symbiosis. While mapping against the publically available transcriptome resulted in low percentages (28.66% aposymbiotic/14.07% symbiotic, Table 2), this provided greater than 20x coverage of the coding sequence for both conditions. One to six percent of the whole-ant RNAseq reads mapped to the ants' principle food source, their fungal garden (*Leucoagaricus*) draft genome (Table 3). Mapping against draft genomes of leaf-cutter garden bacteria, including *Enterobacter*, *Kelbsiella*, *Pantoea*, *Pseudomonas* and *Serratia* draft genomes, all resulted in 0.02% reads mapping or less, as did mapping to proposed symbionts in the genus *Streptomyces*. Low bacterial read numbers are likely a result of polyA selection prior to sequencing, which favors mature eukaryotic reads. In addition, it is likely these low numbers reflect the small fraction these garden-associated species represent as a proportion of the total sample. I also mapped reads back to a composite *Wolbachia* genome, generated by concatenating 4 closed genome sequences, resulting in 0.2 (aposymbiotic) to 1.60% (symbiotic) mapping. Mapping against a composite *Mycoplasma* genome, generated by concatenating 6 closed genomes, resulting in 0.12 (aposymbiotic) to 0.45% (symbiotic) alignments. Mapping against a composite *Pseudonocardia* genome, generated by concatenating 10 draft genome sequences, confirmed reduced *Pseudonocardia* levels in aposymbiotic ants (0.64%) compared with their symbiotic counterparts (4.33%) (Table 3, Table 4). The continued low level of *Pseudonocardia* reads in the aposymbiotic ants may result from low levels of residual of exosymbiont. These reads may also represent the three other associated strains of *Pseudonocardia* reported in association with surface sterilized *Acromyrmex echinatior*, possibly representing gut bacteria (Van Borm *et al*. 2002). While the genome of the particular strain of *Pseudonocardia* strain from the colony used in these experiments has not been sequenced,

mapping against ant associated *Pseudonocardia* genomes gives alignments corresponding with the position of these strains in the whole genome sequence tree. The highest mapping percentages correspond to clade VI *Pseudonocardia* strains, associated with leaf-cutter ant symbionts.

I found 441 genes of 17,227 (or 2.5% genes in the current version of the *Acromyrmex echinatior* genome) were significantly differentially regulated between symbiotic and aposymbiotic ants; of these 78 were downregulated in the aposymbiont while 363 were upregulated. GO term analysis revealed expression changes in only three categories robust enough to remain significant at the p = 0.05 level after Bonferroni correction for multiple testing. These terms included two cellular components—GO:0016021 integral to membrane and GO:0005576 extracellular region and one Biological Process term—GO:0055114 oxidation-reduction processes. While chitin metabolism (GO:0006030) was just shy of statistical significance and chitin catabolism (GO:0006032) also high on the list, two chitinases, were significantly upregulated.

I examined expression in pathways of interest using KAAS automated annotations to detect genes potentially involved in pathways of interest in the *Acromyrmex echinatior* genome. This annotation method discovered complete pathways orthologous to both the mammalian and fruit fly circadian rhythm pathways. Neither clock pathway contained any significantly differentially regulated genes. The KAAS annotator found 14 genes orthologous to the JAK-STAT pathway, none of which were differentially regulated. This method detected orthologs for twenty genes in the TOLL-like immune pathway, only one of which showed significant differential regulation. This upregulation was in TAK1, a gene shared with the NF-Kappa B pathway (Figure 6). The annotation engine found 14 genes in this pathway, two of which were significantly upregulated,

another 9 of which increased in expression 1.5-2.2 fold but were not considered statistically significantly upregulated, 2 genes remaining at approximately the same level and a single gene at a different branch of the pathway decreased in expression slightly. Together this data suggests that the NF-Kappa B showed upregulation. 80 genes from the MAP-K pathway were identified. Of these, 3 showed statistically significant differential regulation, 2 down-regulation and 1 upregulation. Overall pathway equilibrium did not seem to be shifted significantly. While 15 Kruggel-like regulators were identified, none showed significant changes in regulation.

V. DISCUSSION

This study examines changes in gene expression arising due to symbiosis by comparing gene expression in aposymbiotic to symbiotic leaf-cutter ants. Approximately 2.5% (441) of the genes in the *Acromyrmex echinatior* OGS showed significant expression changes. Of these, the majority (363) were upregulated in aposymbiotic ants, while 78 were downregulated. Two cellular component GO terms significantly changed in their expression patterns—GO:0016021 integral to membrane and GO:0005576 extracellular region. Both terms correspond to the location of the symbiont, reflecting symbiotic interactions taking place at the ant-bacterial interface. A single Biological Process term—GO:0055114 oxidation-reduction processes was classified as significant. Poulsen and colleagues demonstrated that *Acromyrmex* ants and their visible symbionts together respire 10-20% more than ants whose exosymbiont was removed (2003). This finding provides independent confirmation of redox changes from the ant side. An additional biological process, chitin metabolism (GO:0006030) was just shy of statistical significance and chitin catabolism (GO:0006032). Chitins have been broadly implicated in symbiosis as Host Associated Molecular Patterns (HAMPs). I found two chitinases, involved in

chitin degradation, are significantly upregulated in symbiotic ants. Since HAMPs would be unnecessary in aposymbiotic ants beyond their critical acquisition period (see Chapter 3), this might account for these changes. Future exploration of this system could provide useful insights into initial differences in expression over the developmental course of the symbiosis.

Sequence data quality examination, including read-length v. probability of error and an examination of the parts of the flow cell itself against read quality suggests that the tags used in this experiment did not create a sequencing bias. Sequenced reads mapped at lower than expected levels to the existing draft genome of the ant *Acromyrmex* echinatior. Further analysis creating a de novo transcriptome and mapping reads there may assist in improving mapping quality. However, control pathway analysis mapping reads to OGS v3.8 genomes seems to confirm the accuracy of the limited reads mapping to this genome build. For example, my analysis found complete genetic pathways homologous to mammalian and fly circadian rhythm pathways. In addition, the pathway showed no differential expression between symbiotic and aposymbiotic ants, as expected since I performed all experiments during the same two-hour window each day.

Although I used polyA selection, bacterial read counts give a window into the role of other players within the leaf-cutter ant symbiosis. *Pseudonocardia*, an exosymbiont, was present at the highest levels of any bacteria detected, making up over 4% of reads in the symbiotic ants. Read numbers declined greatly in the aposymbiotic ants, accounting for less than 1% of reads. Van Borm and colleagues reported 4 strains of *Pseudonocardia* as classified by 16s associated with surface sterilized *Acromyrmex echinatior* (2002). Sandra Andersen's work indicates that the lateral plate *Pseudonocardia* account for one of these strains (in review). If the other three

strains are consistent associates, reads from these strains should also map due to shared homology with the sequenced strains. In contrast, very few *Streptomyces* reads accounting for 0.06% were detected in the symbiotic ant, with an order of magnitude fewer in the aposymbionts. While we cannot rule out that these bacteria may be associated with the exosymbiont biofilm, which they make up a very small fraction of this substrate, and these findings suggest *Streptomyces* are unlikely to play a major role in the exosymbiotic association.

I mapped reads back to five strains of garden associated bacteria. These bacteria are likely to be incidentally present on the ant's surface through ant-garden contact or in the digestive tract, ingested along with the fungal cultivar. All species represented less than 0.02% of reads in the aposymbionts, and less than 0.1% in the symbiotic ants. I also mapped sequences back to *Wolbachia* (0.2-1.6%), an endosymbionts of unknown role in the ants, and *Mycoplasma* (0.12-0.45%), a likely parasite. These read levels were highest in symbiont carrying ants. I found higher levels of *Leucoagaricus* reads mapping in the symbiotic condition (4.33%) compared with the aposymbiotic condition (0.64%). While reads were polyA selected so comparatively few bacteria reads remained, I did observe higher levels of all bacterial species investigated including endosymbionts, parasites and beneficial microbes, in the symbiotic ants. These parallel trends of higher non-self tolerance in symbiotic ants when compared with their aposymbiotic counterparts suggests that the mechanisms involved in symbiont acquisition also have a generalized effect. This might have arisen due to differential regulation in the NF-KappaB pathway, which responds to possible microbial infections and appears to be downregulated in symbiotic ants. No significant differences in expression of the JAK/STAT or TOLL-like immune pathways were detected, suggesting that infection with the symbiont does not trigger a generalized immune

response. However, general upregulation was detected in the NF-KappaB pathway in aposymbiotic ants, suggesting that the host may be tuning down immune function in response to the symbiosis. The apparent trend of increased tolerance to organisms across the phylogenetic spectrum in symbiotic ants suggests that exosymbiont acquisition influences the entire symbiosis and is not restricted to ant-bacterial interactions. Work in weevils has suggests that insects have coevolved tissue specific immune expression patterns and responses to symbionts (Login *et al*. 2011). Future work will help to differentiate systemic from tissue specific effects in the ants will help to elucidate the mechanisms responsible for this trend.

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VII. REFERENCES

Abramowski D, Currie CR, Poulsen M (2011) Caste specialization in behavioral defenses against fungus garden parasites in *Acromyrmex octospinosus* leaf-cutting ants. *Insectes Sociaux*, **58**, 65–75.

Altschul S (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

Andersen SB, Hansen LH, Boomsma JJ (in review) Deconstructing a disease defense symbiosis: Specificity and stability of *Acromyrmex-Pseudonocardia* associations in changing environments. *Molecular Ecology*.

Aylward FO, Burnum KE, Scott JJ, *et al*. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus garden. *ISME J*.

Barke J, Seipke RF, Grüschow S, *et al*. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biology*, **8**, 109.

Belt T (1874) *The Naturalist in Nicaragua*. E. Bumpus, London.

Van Borm S, Billen J, Boomsma JJ (2002) The diversity of microorganisms associated with *Acromyrmex* leafcutter ants. *BMC evolutionary biology*, **2**, 9.

Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*, **34**, 41–58.

Chun CK, Troll JV, Koroleva I, *et al*. (2008) Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-*vibrio* association. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11323–11328.

Cox MP, Peterson DA, Biggs PJ (2010) SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*, **11**, 485.

Foster J, Ganatra M, Kamal I, *et al*. (2005) The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS biology*, **3**, e121.

Fraser CM, Gocayne JD, White O, *et al*. (1995) The Minimal Gene Complement of *Mycoplasma genitalium*. *Science*, **270**, 397–404.

Hsiao N, Kirby R (2007) Comparative genomics of *Streptomyces avermitilis, Streptomyces cattleya, Streptomyces maritimus* and *Kitasatospora aureofaciens* using a *Streptomyces coelicolor* microarray system. *Antonie van Leeuwenhoek*, **93**, 1–25.

Hunter S, Jones P, Mitchell A, *et al*. (2011) InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Research*, **40**, D306–D312.

Klasson L, Walker T, Sebaihia M, *et al*. (2008) Genome evolution of *Wolbachia* strain wPip from the *Culex pipiens* group. *Molecular biology and evolution*, **25**, 1877–1887.

Klasson L, Westberg J, Sapountzis P, *et al*. (2009) The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 5725–5730.

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, **10**, R25.

Liu W, Fang L, Li S, *et al*. (2010) Complete Genome Sequence of *Mycoplasma hyorhinis* Strain HUB-1. *Journal of Bacteriology*, **192**, 5844–5845.

Login FH, Balmand S, Vallier A, *et al*. (2011) Antimicrobial Peptides Keep Insect Endosymbionts Under Control. *Science*, **334**, 362–365.

Mahendra S (2005) *Pseudonocardia dioxanivorans* sp. nov., a novel actinomycete that grows on 1,4-dioxane. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, **55**, 593–598.

Minion FC, Lefkowitz EJ, Madsen ML, *et al*. (2004) The Genome Sequence of *Mycoplasma hyopneumoniae* Strain 232, the Agent of Swine Mycoplasmosis. *Journal of Bacteriology*, **186**, 7123–7133.

Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M (2007) KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic acids research*, **35**, W182–185.

Nyholm SV (2000) From the Cover: Establishment of an animal-bacterial association: Recruiting symbiotic *vibrios* from the environment. *Proceedings of the National Academy of Sciences*, **97**, 10231–10235.

Ohnishi Y, Ishikawa J, Hara H, *et al*. (2008) Genome Sequence of the Streptomycin-Producing Microorganism *Streptomyces griseus* IFO 13350. *Journal of Bacteriology*, **190**, 4050–4060.

Papazisi L (2003) The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain Rlow. *Microbiology*, **149**, 2307–2316.

Poulsen M, Bot ANM, Currie CR, Nielsen MG, Boomsma JJ (2003) Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Functional Ecology*, **17**, 260–269.

Sachs J, Simms E (2006) Pathways to mutualism breakdown. *Trends in Ecology & Evolution*, **21**, 585–592.

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*, **25**, 1105–1111.

Trapnell C, Roberts A, Goff L, *et al*. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, **7**, 562–578.

Westberg J (2004) The Genome Sequence of *Mycoplasma mycoides* subsp. mycoides SC Type Strain PG1T, the Causative Agent of Contagious Bovine Pleuropneumonia (CBPP). *Genome Research*, **14**, 221–227.

Wu M, Sun LV, Vamathevan J, *et al*. (2004) Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS biology*, **2**, E69.

Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology*, **11**, R14.

VIII. FIGURES

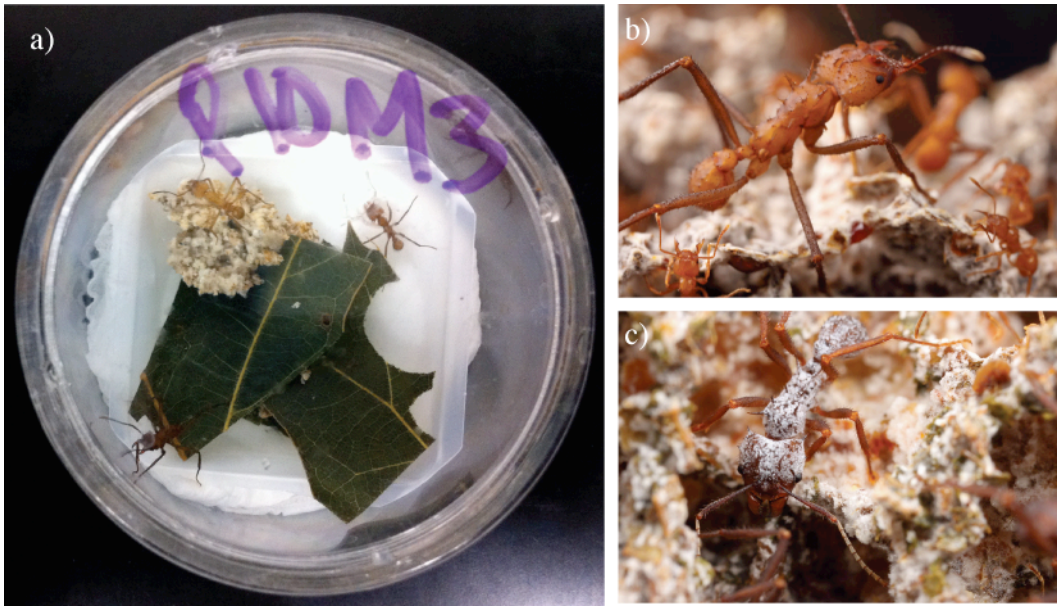


Figure 1. Leaf-cutter ant production. a) Subcolony used to produce focal ants. Colony components include newly eclosed focal ant (lightest) and two major workers (darker), fungus garden and leaf bits. b) Aposymbiotic major. c) Symbiotic major with full covering (sheep). Photo credits: a—Sarah Marsh; b, c—Alex Wild.

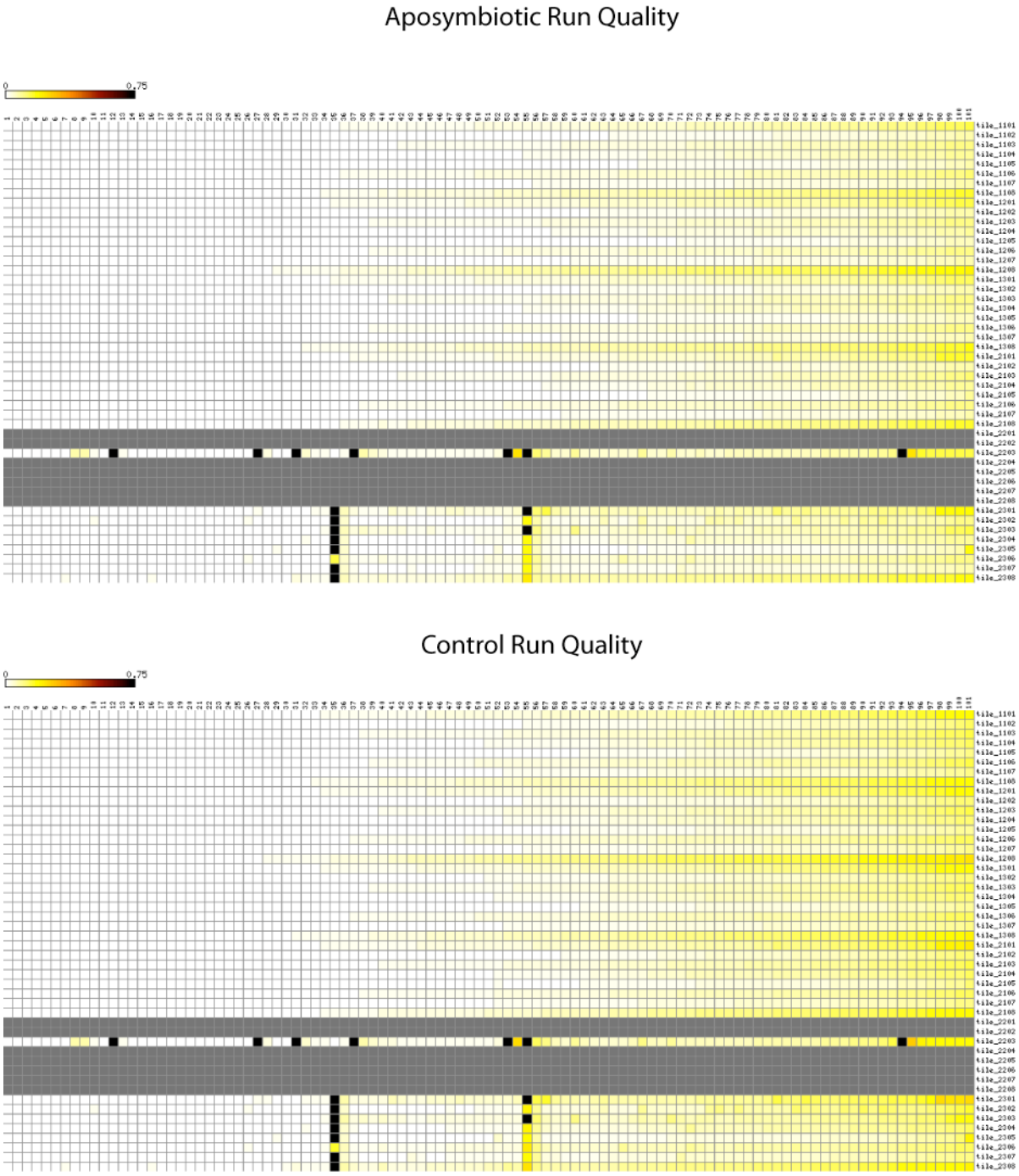


Figure 2 Sequencing Run Quality Heatmap Nucleotide positions are reported from 1 to 100 along the horizontal access. Tile numbers are reported on the vertical access from 1101 to 2308. The color legend runs from high confidence in a base call (white) to low confidence (black). The top heat map shows data for the aposymbiotic condition, while the bottom heatmap the symbiotic or control condition.

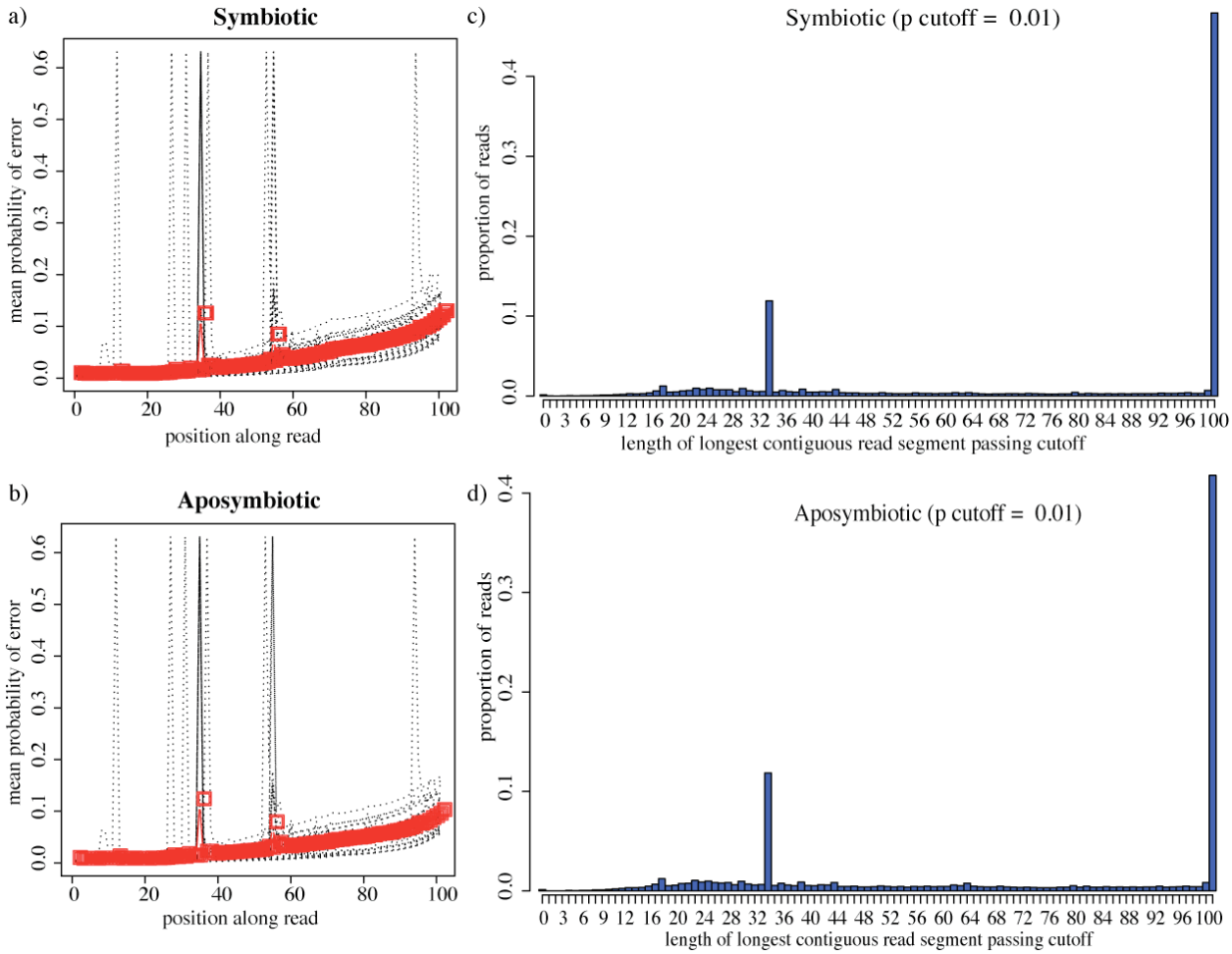


Figure 3. Read Quality. Left panels—dot plots show the mean probability of an error in a nucleotide call on the y axis plotted against red length on the x-axis for a) symbiotic and b) aposymbiotic ants. The red open circles represent run averages, while the black dashes represent tile averages. Right panels—histograms show the length of the longest part of a read whose bases all pass the quality control cutoff (p = 0.01) for c) symbiotic and d) aposymbiotic ants.

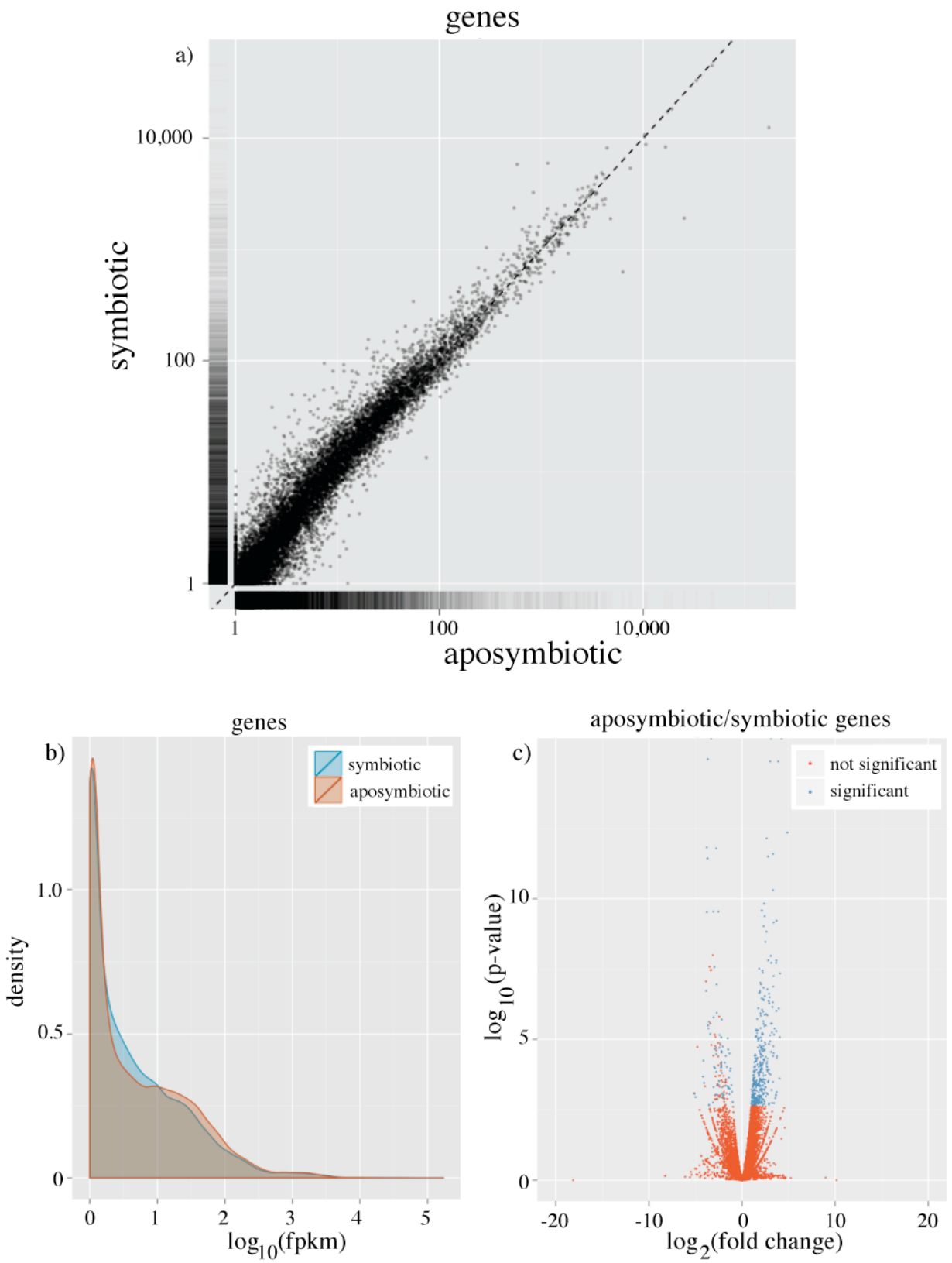

genes
a)
10,000
100
1
symbiotic
1
100
10,000
aposymbiotic
genes
b)
symbiotic
aposymbiotic
1.0
0.5
0
density
0
1
2
3
4
5
log10(fpkm)
aposymbiotic/symbiotic genes
c)
not significant
significant
10
5
0
log10(p-value)
-20
-10
0
10
20
log2(fold change)

Figure 4. Overall Differential Gene Expression Analysis. a) Scatter plot showing gene expression under aposymbiotic (vertical axis) and symbiotic (horizontal axis) conditions. Most genes cluster close to the diagonal line corresponding to similar expression under both conditions. b) Gene expression density plot. Fragments per kilobase of transcriptome per million reads sequence (FPKM) is plotted on the horizontal axis against read density on the vertical axis; symbiotic reads (blue), aposymbiotic reads (red). There is a trend towards upregulation in those genes whose expression did increase significantly. c) Volcano plot showing the $\log_2$ of fold change in gene expression plotted against the $\log_{10}$ of the p-value for this change; points represent individual gene values, statistically significant changes are plotted in blue, others in red.

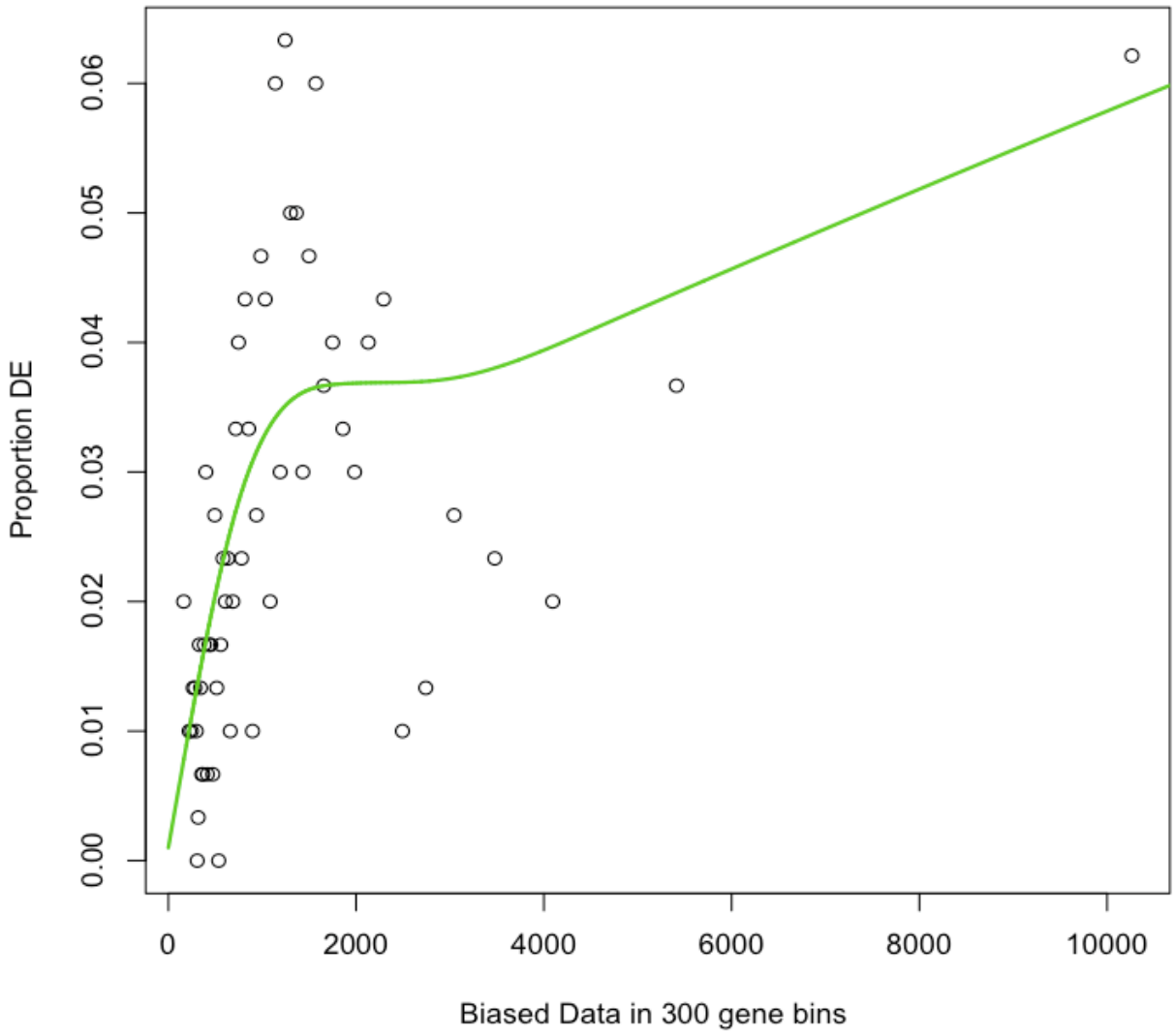


Figure 5. Proportion of differentially expressed genes by length. Each circle represents a bin of 300 genes of similar length. The trend line exemplifies the possibility of bias in detecting expression in longer genes.

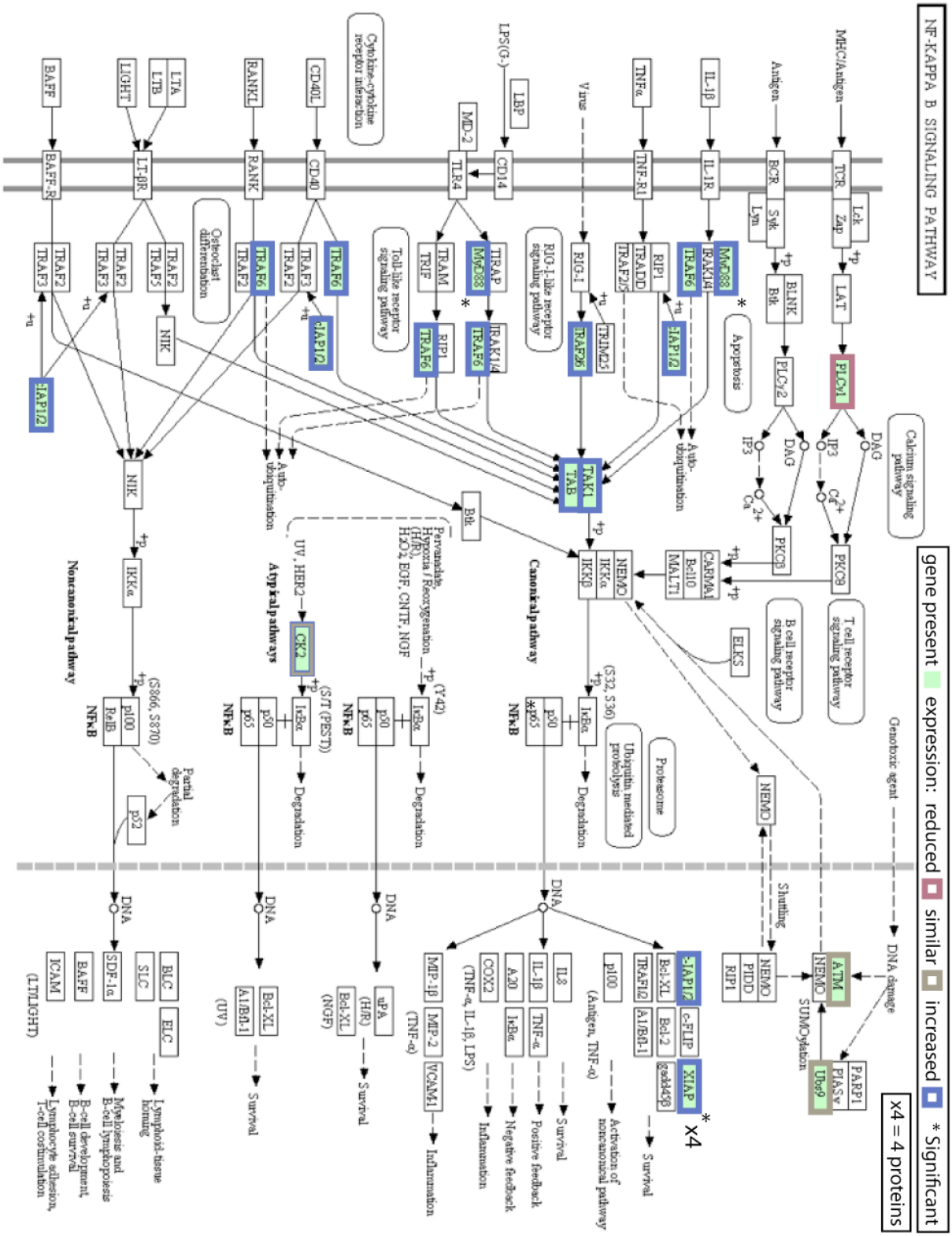

NF-KAPPA B SIGNALING PATHWAY
gene present
expression: reduced
similar
increased
* Significant
x4 = 4 proteins
MHC/Antigen
TCR
Lck
Zap
LAT
PLCγ1
Antigen
BCR
Lyn
Syk
BLNK
Btk
PLCγ2
IL-1β
IL-1R
MyD88
IRAK1/4
IRAK4
IAP1/2
Apoptosis
TNFα
TNF-R1
TRADD
TRAF2/5
RIP1
TRIM25
Virus
RIG-1
TRAF3
LPS(G-)
LBP
CD14
MD-2
TLR4
TRAM
TRIF
TRAP
MyD88
IRAK1/4
IRAK4
TRAF6
RIP1
TRAF6
RIG-I-like receptor signaling pathway
Toll-like receptor signaling pathway
Cytokine-cytokine receptor interaction
CD40L
CD40
TRAF3
TRAF2
TRAF6
IAP1/2
RANKL
RANK
TRAF6
TRAF2
Osteoclast differentiation
LIGHT
LTA
LTB
LT-βR
TRAF2
TRAF3
TRAF2
TRAF5
NIK
BAFF
BAFF-R
TRAF2
TRAF3
IAP1/2
Calcium signaling pathway
DAG
IP3
Ca2+
PKCθ
T cell receptor signaling pathway
B cell receptor signaling pathway
Auto-ubiquitination
TAK1
TAB
CARMA1
Bcl10
MALT1
ELKS
NEMO
IKKα
IKKβ
Canonical pathway
NFκB
p50
*p65
IκBα
(S32, S36)
Degradation
Ubiquitin mediated proteolysis
Proteasome
Genotoxic agent
DNA damage
ATM
NEMO
SUMOylation
PARP1
PIASy
Ubc9
Shuttling
NEMO
PIDD
RIP1
IAP1/2
c-FLIP
XIAP
*x4
Bcl-XL
Bcl-2
A1/Bfl-1
Survival
p100
(Antigen, TNF-α)
Activation of noncanonical pathway
DNA
IL8
Survival
IL-1β
TNF-α
Positive feedback
A20
IκBα
Negative feedback
COX2
(TNF-α, IL-1β, LPS)
Inflammation
MIP-1β
MIP-2
VCAM1
(TNF-α)
Inflammation
uPA
(HIF)
Bcl-XL
(NGF)
Survival
Bcl-XL
A1/Bfl-1
(UV)
Survival
NIK
IKKα
(S866, S870)
p100
RelB
NFκB
Partial degradation
p52
Noncanonical pathway
Atypical pathways
Auto-ubiquitination
Btk
Peroxynitrite, Hypoxia / Reoxygenation (HIR), H2O2, EGF, CNTF, NGF
(Y42)
IκBα
Degradation
(S/T (PEST))
UV, HER2
CK2
IκBα
Degradation
DNA
p50
p65
NFκB
DNA
p50
p65
NFκB
DNA
SDF-1α
BAFF
ICAM
(LTβ,LIGHT)
BLC
SLC
ELC
Lymphoid-tissue homing
Myelopoiesis and B-cell lymphopoiesis
B-cell development, B-cell survival
Lymphocyte adhesion, T-cell costimulation

Figure 6. NF-Kappa B pathway in *Acromyrmex echinatior*. Diagram showing the conserved NF-Kappa B pathway, highlighting those genes confirmed *in silico* in the ant. Although only two genes (MyD88 and XIAP4) are significantly upregulated (from no expression and a 3.5 fold increase), many genes were upregulated 1.5 to 2.2 fold even though this upregulation was not statistically significant. Of interest, the pathway is missing some intermediates, however the first steps (MyD88) and the final steps (XIAP4) are significantly increased. Also of note, four different genes all map to XIAP4. One significantly, the others 1.2-2.2 fold.

IX. TABLES

Table 1. Illumina Run Quality Assessment. This table presents fastq file read quality statistics including the index (tag sequence), percentage PF (percentage of reads receiving a Phred quality scores), read number, percentage of raw clusters per lane (assesses tag bias), percentage of perfect index reads, percentage of one mismatch index reads, Percentage of Bases ≥Q30 (PF) (corresponds to an error probability of 0.1%), and Mean Quality Score (PF).

	Aposymbiotic	**Symbiotic**
Index	ATCACG	GATCAG
Yield (Megabases)	5,021	4,713
Yield (Gigabytes)	12.0	11.3
Percentage PF	100	100
Read number	49,714,198	46,663,620
Percentage of raw clusters per lane	33.05	31.02
Percentage Perfect Index Reads	96.7	96.39
Percentage One Mismatch Index Reads	3.3	3.61
Percentage of Bases with ≥Q30 (PF)	87.27	85.03
Mean Quality Score (PF)	34.4	33.53

Table 2. Read Quality. Statistics describing processed reads. Bold parameters used for subsequent analyses. Legend: Apo-aposymbiotic, Sym-symbiotic.

		10%		5%		**1%**		0.1%	
		Apo	Sym	Apo	Sym	Apo	Sym	Apo	Sym
25bp	Trimmed (Gb)	10.2	9.4	10.0	9.2	**9.6**	**8.8**	8.1	7.3
	>25 bp (Gb)	10.1	9.3	9.9	9.1	**9.3**	**8.5**	7.4	6.5
	mean read length	79.99	78.79	79.38	77.91	**77.04**	**74.85**	68.43	64.52
	median read length	101	101	101.0	101	**101**	**94**	69	62
	read #	48,780,578	45,573,593	48,491,328	45,311,575	**46,920,905**	**43,609,310**	40,328,385	36,930,824
	mapped reads	11,840,827	6,129,688	13,709,376	6,198,511	**13,447,315**	**6,136,413**	11,840,828	5,524,647
	% reads mapped	29.36%	13.45%	28.27%	13.68%	**28.66%**	**14.07%**	29.36%	14.96%
	reads mapping ≥2x	1,389	532	1550	589	**2004**	**712**	2,606	938
50bp	Trimmed (Gb)	10.6	9.8	10.5	9.2	10.0	8.8	8.5	7.3
	>50 bp (Gb)	9.1	8.3	8.7	7.6	8.0	6.9	5.8	4.8
	mean	96.16	94.77	95.74	94.41	94.30	92.03	87.59	83.42
	median	101	101	101	101	101	101	101	90
	read #	37,631,624	34,814,352	36,158,943	33,185,582	33,481,030	30,664,025	26,129,746	22,934,732
	mapped reads	10,169,206	4,422,173	9,777,062	4,251,806	9,153,278	3,987,635	7,291,925	3,196,855
	% reads mapped	27.02%	12.70%	27.04%	12.81%	27.34%	13.00%	27.91%	13.94%
	reads mapping ≥2x	94	23	96	21	91	24	68	20
75bp	Trimmed (Gb)	10.2	9.4	10.0	8.8	9.6	8.8	8.1	7.3
	>75 bp (Gb)	7.7	6.9	7.5	5.9	6.8	5.9	4.4	3.3
	mean	99.78	99.37	99.72	99.31	99.11	98.42	92.7	95.82
	median	101	101	101	101	101	101	101	101
	read #	32,163,379	28,985,893	31,405,655	28,139,027	28,485,698	24,762,140	18,589,430	14,302,408
	mapped reads	8,636,638	3,743,008	8,434,061	3,654,648	7,681,794	3,332,398	4,948,746	2,153,898
	% reads mapped	26.85%	12.91%	26.86%	12.99%	26.97%	13.46%	26.62%	15.06%
	reads mapping ≥2x	71	14	70	14	61	14	47	11

Table 3. Sequence mapping by species.

Species	Description	Aposymbiotic read #	%	Symbiotic read #	%
Acromyrmex echinatior	Leaf-cutter ant	13,449,319	96.07	6,137,125	66.57
Leucoagaricus	Fungal cultivar	420,183	3.00	2,478,579	26.89
Wolbachia	Endosymbiont	28,279	0.20	148,346	1.61
Mycoplasma	Parasite	16,719	0.12	41,439	0.45
Pseudonocardia	Exosymbiont	90,112	0.64	401,229	4.33
Streptomyces	Proposed exosymbiont	642	0.00	5,753	0.06
Enterobacter	Garden associated bacteria	2,054	0.01	9,040	0.10
Klebsiella	Garden associated bacteria	2,054	0.01	9,225	0.10
Pantoea	Garden associated bacteria	2,040	0.01	8,937	0.10
Pseudomonas	Garden associated bacteria	2,212	0.02	10,414	0.11
Serratia	Garden associated bacteria	2,229	0.02	10,199	0.11

Table 4. *Pseudonocardia* species mapping. Below are the number of reads mapping to each species of *Pseudonocardia* along with the percent of the total reads assigned.

			Aposymbiotic		Symbiotic	
Species	**Description**	**Clade**	**read #**	**%**	**read #**	**%**
Pseudonocardia	All		83,176	0.18%	398,711	0.91%
Pseudonocardia dioxanivorans	Free-living		1,742	0.00%	22,642	0.05%
Pseudonocardia saturnea	Free-living		4,561	0.01%	35,623	0.08%
Pseudonocardia sp. AL050513-04	*Tracymyrmex zeteki* associated	I	1,753	0.00%	22,318	0.05%
Pseudonocardia sp. CC030328-06	*Mycerotes parallelus* associated	III	1,714	0.00%	23,942	0.05%
Pseudonocardia sp. CC011120-04	*Apterostigma dentigerum* associated	IV	10,399	0.02%	40,624	0.09%
Pseudonocardia sp. CC031212-01	*Acromyrmex echinatior* associated	IV	1,754	0.00%	31,878	0.07%
Pseudonocardia sp. P1	*Acromyrmex octospinosus* associated	IV	1,746	0.00%	30,585	0.07%
Pseudonocardia sp. CC031210-22	*Acromyrmex octospinosus* associated	VI	2,796	0.01%	40,611	0.09%
Pseudonocardia sp. AL041002-03	*Tracymyrmex cornetzi* associated	VI	47,515	0.10%	225,534	0.52%
Pseudonocardia sp. P2	Acromyrmex octospinosus associated	VI	51,605	0.11%	327,940	0.75%

Table 5. Biological Process Gene Ontology terms associated with differential expression.
Terms in Bold remained statistically significant at the p = 0.05 level after Bonferroni correction.

GO id	Description	p-value
GO:0055114	**oxidation-reduction process**	**0.000032**
GO:0006030	chitin metabolism	0.00037
GO:0015986	ATP synthesis coupled proton transport	0.003
GO:0006506	glycosylphosphatidylinositol biosynthesis	0.010
GO:0005975	carbohydrate metabolism	0.013
GO:0030041	actin filament polymerization	0.019
GO:0016559	peroxisome proliferation	0.023
GO:0009116	nucleoside metabolism	0.024
GO:0006629	lipid metabolism	0.024
GO:0007050	cell cycle arrest	0.024
GO:0006672	ceramide metabolism	0.028
GO:0006032	chitin catabolism	0.028
GO:0009156	ribonucleoside monophosphate biosynthesis	0.032
GO:0030328	prenylcysteine catabolism	0.034
GO:0006261	DNA-dependent DNA replication	0.034
GO:0006368	transcription elongation from RNA polymerase II promoter	0.035
GO:0007269	neurotransmitter secretion	0.035
GO:0006425	glutaminyl-tRNA aminoacylation	0.035
GO:0006915	apoptotic process	0.036
GO:0006807	nitrogen compound metabolism	0.039
GO:0006952	defense response	0.041
GO:0006414	translational elongation	0.044

Table 6. Cellular Component Gene Ontology terms associated with differential expression.
Terms in Bold remained statistically significant at the p = 0.05 level after Bonferroni correction.

GO id	Description	p-value
GO:0016021	**integral to membrane**	**0.00029**
GO:0005576	**extracellular region**	**0.00041**
GO:0005789	endoplasmic reticulum membrane	0.010
GO:0005787	signal peptidase complex	0.033
GO:0008023	transcription elongation factor complex	0.035
GO:0016533	cyclin-dependent protein kinase 5 holoenzyme complex	0.036

Table 7. Molecular Function Ontology terms associated with differential expression. No terms remained statistically significant at the p = 0.05 level after Bonferroni correction.

GO id	Description	p-value
GO:0015078	hydrogen ion transmembrane transporter activity	0.00018
GO:0043169	cation binding	0.00035
GO:0008061	chitin binding	0.00037
GO:0020037	heme binding	0.00053
GO:0005506	iron ion binding	0.00057
GO:0009055	electron carrier activity	0.0026
GO:0005344	oxygen transporter activity	0.0070
GO:0008234	cysteine protease activity	0.011
GO:0046983	protein dimerization activity	0.011
GO:0003824	enzyme activity	0.012
GO:0004197	cysteine-type endopeptidase activity	0.014
GO:0004497	monooxygenase activity	0.014
GO:0042302	structural constituent of cuticle	0.015
GO:0008168	methyltransferase activity	0.015
GO:0004252	serine-type endopeptidase activity	0.019
GO:0004852	uroporphyrinogen-III synthase activity	0.027
GO:0004568	chitinase activity	0.028
GO:0022857	transmembrane transporter activity	0.030
GO:0003746	translation elongation factor activity	0.030
GO:0004731	purine-nucleoside phosphorylase activity	0.031
GO:0004108	citrate (Si)-synthase activity	0.034
GO:0004505	phenylalanine 4-monooxygenase activity	0.034
GO:0004611	phosphoenolpyruvate carboxykinase activity	0.035
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) activity	0.035
GO:0017076	purine nucleotide binding	0.035
GO:0004819	glutamine-tRNA ligase activity	0.035
GO:0017134	fibroblast growth factor binding	0.036
GO:0016534	cyclin-dependent protein kinase 5 activator activity	0.036
GO:0008081	phosphoric diester hydrolase activity	0.049

CHAPTER 5. EXPLORING LEAF-CUTTER ANT-*PSEUDONOCARDIA* INTERACTIONS ACROSS SCALES

I. Overview

Symbiosis, the living together of unlike organisms (de Bary 1879), has been and remains critically important to the evolution of life on Earth. Virtually all animals host beneficial microbial symbionts, some of which are critical for normal host development as well as for both organisms' survival. Leaf-cutter ants farm a fungal cultivar as their primary nutrient source. The ants also carry a specialized Actinobacterial exosymbiont, mainly in the genus *Pseudonocardia*, which produces antifungal compounds that inhibit *Escovopsis*, a parasite that specializes on the ants' fungal garden. This symbiotic system has culminated in some of the most dominant herbivores on Earth today.

The success of a mutualism depends on hosts' ability to properly acquire and maintain appropriate symbionts. In this dissertation, I use the leaf-cutter ant symbiosis as a model to examine host-beneficial microbe interactions. I begin with a general overview reviewing the theoretical basis of symbiont transmission. Next, I delved into the mechanistic details of successful symbiont acquisition and maintenance. These processes facilitate the stability needed for long-term coevolution, and indeed the evolution of mutualism.

In chapter two, I explored the breadth of the leaf-cutter ant-*Pseudonocardia* association. Among the leaf-cutting ants, the bacterial association is clear in those ant species that carry visible loads of exosymbionts (mainly in the genus *Acromyrmex*), but remains unclear in those that do not (mainly ants in the genus *Atta*). I employed a variety of methods to isolate *Pseudonocardia* and other actinobacteria from *Atta cephalotes*. These experiments focused on five lab-maintained

colonies. I was able to isolate *Pseudonocardia* from all five colonies, in addition to ten additional lab colonies. Phylogenetic analysis demonstrated that three of the strains isolated cluster with the visible strains isolated from *Acromyrmex* colonies. This suggests that the leaf-cutter ant-*Pseudonocardia* association may be maintained, at least in some colonies. In addition, I isolated two apparently novel clades of *Pseudonocardia* bacteria. Future work will be needed to show the location of these strains and possible functions.

In chapter 3, I explored the details of within-colony symbiont transmission. *Acromyrmex* leaf-cutter ants carry a vertically transmitted exosymbiont (Currie *et al*. 1999). However, little was known about specific mechanisms responsible for transmission. I performed subcolony experiments to explore the components necessary for successful acquisition. These manipulations show that ants of a specific caste (majors) carrying *Pseudonocardia* are essential for transmission to take place. I also manipulated subcolonies to explore importance of timing on acquisition. I found that there is a short critical window of a few hours during which the ants can acquire *Pseudonocardia*. If transmission does not occur during this period, the ants will not acquire their exosymbionts. This critical window may serve to minimize infection by other strains of bacteria and to help maintain partner fidelity within the symbiosis.

In chapter 4, I explored the genetic basis of symbiosis. To do this I examined gene expression in *Acromyrmex echinatior* leaf-cutting ants ten days post-eclosion, comparing ants normally colonized by their exosymbiont (holosymbionts) against ants prevented from normally acquiring their exosymbiont (aposymbionts). This comparison found 441 significantly differentially expressed genes, showing greater than a two-fold difference in expression change when normalized for reads sequenced for each condition. Within these genes, more (363) were

overexpressed than (78) underexpressed. Gene ontology (GO term) analysis found only three categories significantly changed in differential expression at the $p = 0.05$ level after Bonferroni correction for multiple testing. These categories included two cellular component terms—GO:0016021 integral to membrane and GO:0005576 extracellular region (corresponding with the location of the symbiont) and one Biological Process term—GO:0055114 oxidation-reduction processes. However, chitin metabolism (GO:0006030) showed a non-significant trend for differential regulation, as did chitin catabolism (GO:0006032). Chitin and its derivatives can play an important role in host-symbiont recognition. These surface polymers are common at the host-symbiont physical interface and often function as host associated molecular patterns or HAMPs (reviewed in Chaston & Goodrich-Blair 2010). In this light, it is not surprising that an uncolonized host would not maintain recognition markers for its absent partner. Pathway analysis investigating putative immune gene function found differential regulation in the NF-Kappa B pathway alone. This pathway is canonically involved in bacterial antigen recognition and governing responses fighting microbial infection (reviewed in Hayden & Ghosh 2011). It appears to be upregulated in the aposymbiotic host. Concomitantly, all host-associated microbes, be they from garden bacteria, endosymbionts or possible pathogens show fewer reads. This suggests that this host reduces general microbial defenses in order to accommodate its symbiont. This difference appears to be systematic, however, future studies isolating gene expression specifically in colonized tissue would reveal whether this difference is system-wide or tissue specific (c.f. Login *et al*. 2011). Previous studies using Expressed Sequence Tags (ESTs) to look at gene expression in symbiont colonization in the *Euprymna-Vibrio* symbiosis found similar differential regulation in the NF-KappaB pathway (Chun *et al*. 2008). However, other genes apparently active in that symbiosis were not found to be differentially regulated in the leaf-cutter

ant symbiosis. This suggests a conserved pathway across symbiosis, however dissimilarities could arise due to experimental timing (*e.g.* how long after the initiation of colonization).

This dissertation examines the leaf-cutter ant-*Pseudonocardia* mutualism over three scales: at the species, individual host, and, finally, molecular-genetic level. More specifically, I explore from the fastidious nature of *Pseudonocardia* in culture, to the particular conditions and timing needed for proper symbiont acquisition in ants, to the regulatory changes triggered by symbiont presence, specificity emerges as a common theme. While this thesis builds on current understanding of the mechanisms used to establish and maintain specificity within symbioses, much future work remains.

The genomics revolution has spurred traditional symbiotic model systems to become genetic model systems including leaf-cutting ants, while at the same time traditional genetic model systems, such as zebra fish (*Danio rerio*) and fruit flies (*Drosophila melanogaster*) have recently been turned to study symbiosis (Nygaard *et al*. 2011; Suen *et al*. 2011; Rawls *et al*. 2004; Bates *et al*. 2006; Ryu *et al*. 2008; Wong *et al*. 2011). Researchers are taking advantage of molecular techniques in a wide variety of symbiotic models, including the Hawaiian bobtailed squid (*Euprymna scolopes*), earthworm (*Eisenia fetida*) and medical leeches (*Hirudo* sp.) (c.f. Chun *et al*. 2008; Graf *et al*. 2006; Lund *et al*. 2010). All of these systems bring the potential for powerful evolutionary insights to the study of symbiosis. The future promises insights gleaned from these models, transitioned to non-model organisms including humans. The recent surge of scientific interest in beneficial microbes coupled with the many developing model systems available will in turn continue to greatly facilitate comparative work to elucidate more of the first principles of symbiosis as well as practical applications from this work.

II. References

de Bary HA (1879) *Die Erscheinung der Symbiose*. Karl J. Trubner, Strasburg.

Bates JM, Mittge E, Kuhlman J, *et al*. (2006) Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental biology*, **297**, 374–386.

Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*, **34**, 41–58.

Chun CK, Troll JV, Koroleva I, *et al*. (2008) Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-*vibrio* association. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11323–11328.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.

Graf J, Kikuchi Y, Rio RVM (2006) Leeches and their microbiota: naturally simple symbiosis models. *Trends in microbiology*, **14**, 365–371.

Hayden MS, Ghosh S (2011) NF-κB in immunobiology. *Cell Research*, **21**, 223–244.

Login FH, Balmand S, Vallier A, *et al*. (2011) Antimicrobial Peptides Keep Insect Endosymbionts Under Control. *Science*, **334**, 362–365.

Lund MB, Davidson SK, Holmstrup M, *et al*. (2010) Diversity and host specificity of the *Verminephrobacter*-earthworm symbiosis. *Environmental microbiology*, **12**, 2142–2151.

Nygaard S, Zhang G, Schiott M, *et al*. (2011) The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. *Genome research*, **21**, 1339–1348.

Rawls JF, Samuel BS, Gordon JI (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 4596–4601.

Ryu J-H, Kim S-H, Lee H-Y, *et al*. (2008) Innate immune homeostasis by the homeobox gene Caudal and commensal-gut mutualism in *Drosophila*. *Science*, **319**, 777–782.

Suen G, Teiling C, Li L, *et al*. (2011) The Genome Sequence of the Leaf-Cutter Ant *Atta cephalotes* Reveals Insights into Its Obligate Symbiotic Lifestyle RID F-1026-2011. *Plos Genetics*, **7**, e1002007.

Wong CNA, Ng P, Douglas AE (2011) Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environmental microbiology*, **13**, 1889–1900.

APPENDIX 1. TEACHING AND LEARNING PORTFOLIO

by

Sarah Marsh
Genetics Graduate Training Program
Department of Bacteriology

July 12th, 2012

This portfolio submitted in partial fulfillment of the requirements for the
Delta Certificate in Research, Teaching, and Learning.

Delta Program in Research, Teaching, and Learning
University of Wisconsin-Madison

The Delta Program in Research, Teaching, and Learning is a project of the Center of the Integration of Research, Teaching, and Learning (CIRTL—Grant No. 0227592). CIRTL is a National Science Foundation sponsored initiative committed to developing and supporting a learning community of STEM faculty, post-docs, graduate students, and staff who are dedicated to implementing and advancing effective teaching practices for diverse student audiences. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

TEACHING PORTFOLIO INTRODUCTION

This portfolio is an effort to represent my own personal development as a teacher over my time as a graduate student at the University of Wisconsin-Madison (UW). Over the last few years, my teaching style has been greatly influenced by participating in the Delta Program in Research, Teaching and Learning, a University of Wisconsin learning community designed to educate future faculty in teaching and learning. The main foundations of this program are three pillars: 1) teaching as research, 2) learning communities, and 3) learning-through-diversity.

I came to Delta with a long history as both a learner and a teacher. I have been a student in diverse disciplines ranging from anthropology to oceanography to biology and even yoga for many years. This wide breadth of learning experiences gives me insight into the teaching and learning styles that worked best for me and for my classmates. As a teaching assistant, I had worked with diverse classes from an animal behavior lab of a dozen students to sections of 25-45 in a genetics course of over 300 total students. In these courses, I have run small discussions and graded assignments as well as lectured to larger groups and written exam questions. In addition, I had the unique opportunity to observe different professors' styles—from highly critical to very laid back to rigidly structured, perhaps most importantly where these styles worked and where they failed.

As an aspiring scientist I was intrigued by the idea of **Teaching-As-Research**. I look to studies and data to inform most aspects of my life. Looking at teaching and learning from this perspective, then adapting my own practices accordingly was a natural approach. I first incorporated information from teaching-as-research studies into my own teaching repertoire. I

eventually went on to develop, implement and analyze data from a case study designed to allow students to explore the main principles behind evolution by natural selection in microorganisms. Wherever I teach next, teaching-as-research will certainly influence my classroom.

The Delta Program also emphasizes the importance of *establishing and nurturing* **learning communities**. The feedback I have received from my Delta classmates and other participants has been extremely useful in developing my teaching style. In addition, having a supportive peer-network can foster creative approaches to the larger problems facing all of modern academia. I have felt fortunate to take part in Delta Roundtable dinners. These experiences expanded both my network of teaching collaborators while at the same time providing insights into topics from the Wisconsin Idea to bridging the achievement gap to international teaching.

Finally, Delta emphasizes **learning-through-diversity**. The program taught me to first recognize the many types of potentially "hidden" diversity in the people in any college classroom—from those who are the first in their families to go to college to students from rural settings, not to mention the hundreds of possible majors available at the University of Wisconsin from traditional majors like Biology to specialized majors including Turf Grass. Once I had a greater appreciation of this diversity, Delta encourages teachers to see it as an advantage rather than a hindrance. All students benefit from clear presentation and repetition in different modes. The following portfolio was designed to highlight my development as a future faculty member.

TEACHING STATEMENT

I believe that inspirational teachers have the ability to make a significant impact on their students' lives. This kind of teacher ignited my own desire for teaching and studying evolutionary biology. In addition to enthusiasm, good teaching requires thoughtful course design coupled with frequent student assessment and adjustment. Instruction does not end at the classroom door. Active mentoring is an important part of being a good teacher. In the end, students will hopefully take away not only knowledge and methodology, but also passion and inspiration.

I believe that planning a high-quality course starts with pinpointing the most important principles from a given discipline, then designing assessments around those ideas. This **backward design** process includes developing student-centered interactive material learners can access and utilize outside the classroom to dispel common misconceptions surrounding a topic and designing assignments that encourage students to prepare before coming to class.

I **present the same material in different ways**. This not only caters to students with different learning styles, but also provides the class with some of the repetition necessary to learn new material on a deeper level. Presenting the most important concepts using multiple methods can also act as a back-up plan, giving you options for smooth transitions even when one approach fails.

Engaging students requires creating a comfortable class environment where **students are accountable partners in the learning process**. I frequently evaluate students' understanding and ability to use new information through **formative and summative assessments** to

maximizing student learning. When students falter, frequent feedback assures a quick adjustment. I have found using iClicker questions in the classroom very useful. For example, when teaching a unit on evolution by natural selection, the students were hung-up on the concept of variation within populations. To me this is a straightforward concept, so without the instant feedback, I would have plowed ahead, unaware I had lost them.

Mentoring students is a key component of the scientific education process. I believe that regular open communication is the foundation for this learning-partnership. Assessing and adapting to the student's changing needs over the course of the interaction is critical to providing them with tailored support. My goal is to teach each student the fundamentals of hypothesis driven scientific research, while allowing them to develop their own ideas and questions to smooth the transition from completing small lab tasks to their own projects. I recently research-mentored a student working in the Currie lab in her senior year at the University of Wisconsin. She had experience working in two other labs and was very enthusiastic, but had no experience working with our non-model organism. After orienting her to the leaf-cutter ant symbiosis and associated lab techniques, I was able to give her a lot of independence. I was present for questions but gave her space to succeed without being micro-managed. I enjoy c**ultivating one-on-one mentoring relationships**. In my view this is a critical step in fostering independent thinkers.

As with all skills, teaching improves with practice. I pursued a certificate in the University of Wisconsin-Madison Delta Program in Research, Teaching and Learning to develop my teaching skills. This program is designed to help educate future professors in teaching and learning. As a participant, I took courses designed to enhance my teaching, including a seminar on teaching

large classes and a course on international teaching. I also completed a teaching-as-research internship, designing a current events based case study to reinforce the fundamental principles of evolution by natural selection in a microbial evolution context. I have enjoyed my teaching and mentoring experiences and am looking forward to a career as a scientist and an educator.

TEACHING ARTIFACT OVERVIEW

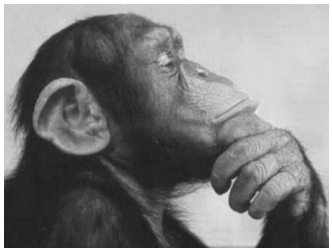
The following sections of the portfolio were built around a series of teaching artifacts. The first four artifacts were all drawn from experiences connected to a lecture on the origin of life I taught in the spring of 2011 and again in 2012. This class-session was part of a course on Microbial Ecology, Evolution and Diversity aimed at upper level microbiology majors. **Artifact one** presents a selection of slides from my teaching, highlighting many of the different teaching techniques I incorporate into a class session to connect with students with a variety of learning styles and diverse backgrounds. **Artifact two** includes a screen-shot of the course Moodle. This website provides an example of the many interactive tools I was able to make available to the students in this course. This allowed me to provide extra material for both students struggling to understand basic concepts as well as those wishing to go beyond the in-class activities. **Artifact three** is a teaching evaluation, completed by one of my Delta seminar on Teaching Large Classes classmates. This evaluation showcases the importance of my peer group. Interacting with the learning community fostered by participating in Delta both formally and informally was one of the most valuable parts of the program experience. **Artifact four** came from an experimental classroom observation project I participated in developed my Matthew Hora. The goal of the study was to provide teachers with useful, standardized feedback on their classroom activities. This artifact is a figure generated from this observation, showing the flow of my teaching. It emphasizes my effort to keep the students actively involved over the course of the class-time.

Artifact five is a student email I received while TAing Genetics 466. This student emailed to ask a question from a related class. I included it to highlight my effort to create a comfortable learning-environment.

Artifact six is the summative report for my teaching-as-research project. This project centered around developing, implementing and evaluating a current-events-based case study to teach the principles underlying evolution by natural selection to microbiology students.

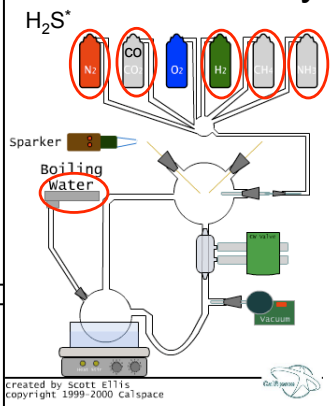
1

Q. What is life?



2

Miller-Urey Experiments

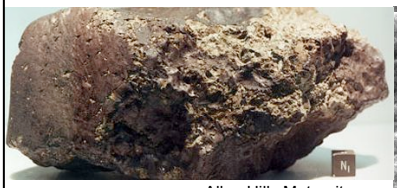


- Try them yourself
  http://www.ucsd.tv/miller-urey
- Miller explains
  -why
  -set-up

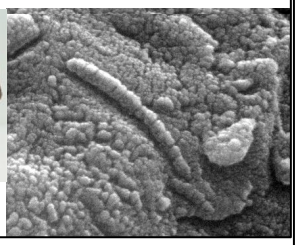
3

Life on Mars?

- New focus ➔ microbial life
- Meteorite evidence
  – All could be produced by abiotic mechanisms



Allen Hills Meteorite



Clicker Question

4

How many times did life originate on Earth?

a. 0
b. 1
c. 10
d. 100
e. 1,000

What might limit this process?

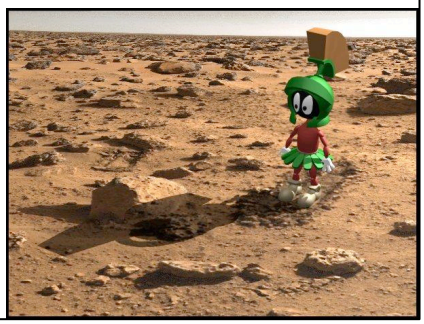
Clicker Question

5

Is there life on Mars?

a. yes
b. no

As microbiologists, what data would you want to make your case?



ARTIFACT#1 ORIGIN OF LIFE SLIDE SAMPLE

A#1 Description

Above are a selection of slides from the Origin of Life lecture discussed above. I chose these few to illustrate the variety of teaching modalities I try to incorporate into my classroom teaching.

Slide one. This slide poses the question "What is life?". I introduce this to the students as an easy way to start the lecture—after all we all intuitively think we know how to define life. I give the students a few minutes to discuss this with their neighbors. Once I bring the class back together, I make a list of the qualities they come up with on the board and we discuss problems with a strict definition of life.

Slide two. This slide contains both a link to a video, featuring Henry Miller explaining the apparatus he used to test the hypothesis that the constituents of the early earth environment could produce simple amino acids. I also provide a link to a website on the Moodle site associated with this course (see Artifact#4).

Slide three. This slide contains a meteorite with possible evidence of life on Mars. This meteorite was tested by a summer Research Experience for Undergraduates student, who described evidence for life on this rock. For her role in the work the student was an author on a paper published in the prestigious journal Science. I tell this story to let the students know what potential lab work they may be able to do in the future. This hook helps grab their attention.

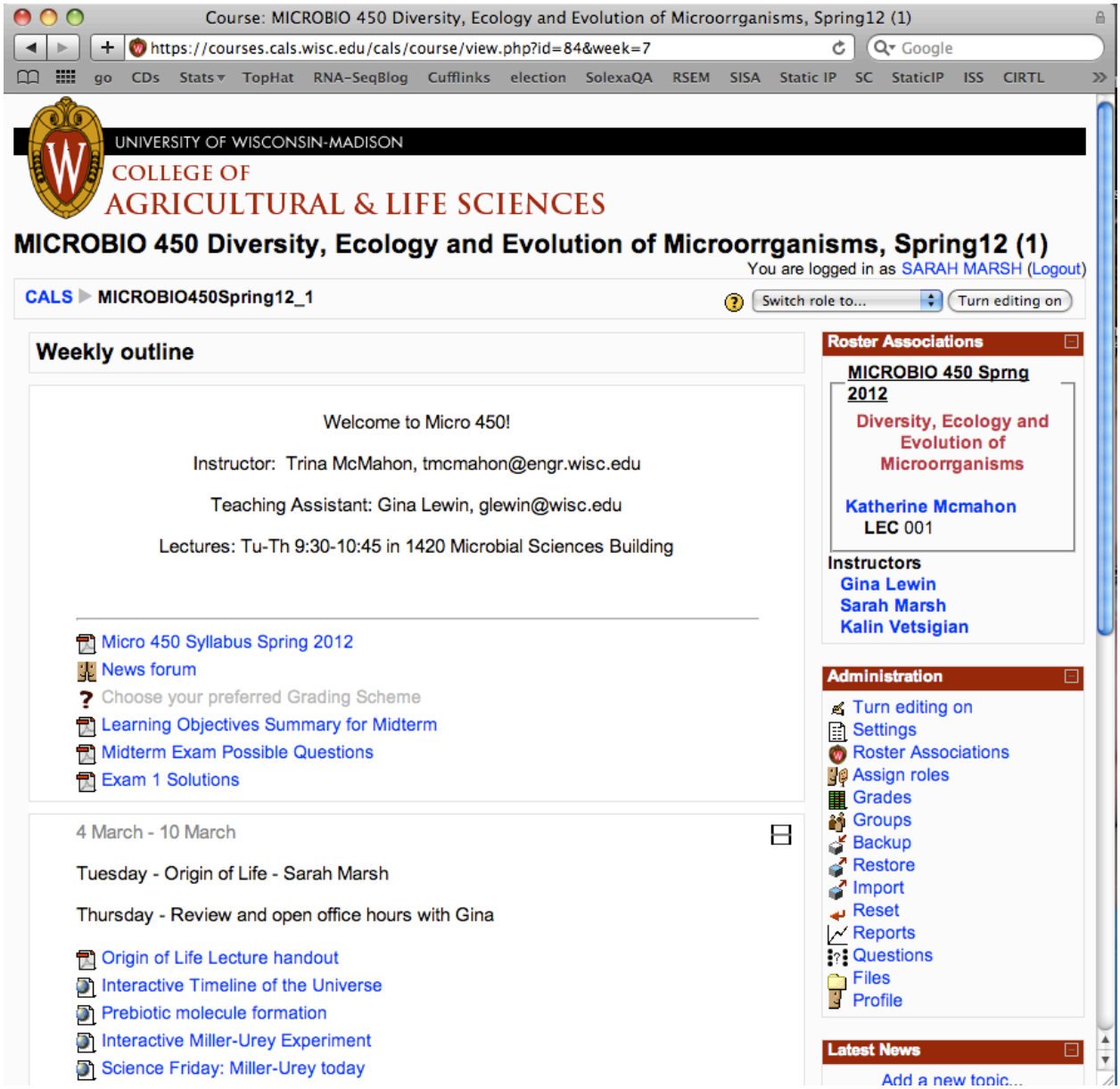
Slide four. This slide asks them to apply what they've learned over the course of the lecture to speculate about the number of origins of life. This question doesn't have a correct answer (at least not that we can articulate). I give them a minute to discuss with their neighbors, then we

discuss the main points as a group—reviewing the main points of the conditions required for the origin of life.

Slide five. After covering more evidence for and against life on Mars, slide 5 asks the students if they believe there is life on Mars. I start this section of the lecture asking the same question, then return to this question to see if opinions have changed (and if they are thinking more about microbial possibilities). I finish the lecture having them design additional experiments to prove their assertion (for or against life on Mars).

A#1 Reflection

Students in any college classroom will represent diverse groups—from obvious gender and ethnic differences to subtler differences in socioeconomics and classroom preparation. These differences will also encompass learning style and preferences. I aspire to use a variety of learning and teaching modalities not only to reach, but also to take advantage of the student diversity.



ARTIFACT#2 MICRO450 MOODLE

A#2 Description

The Moodle website from the Origin of Life lecture discussed above. Note the numerous links. The first corresponds to a set of the slides, so students can spend time in the lecture thinking about the concepts rather than frantically copying down text and figures. To encourage students to come to class and pay attention, I leave blanks spaces in some slides that they need to fill-in during the lecture. I learned this teaching trick from my advisor, Cameron Currie. The second link is to an interactive timeline of the universe, perhaps helpful in orienting them to the scale of time we cover in the lecture. The third link is an interactive animation that I also play in class and have the class vote about the outcomes they expect. The forth link is an interactive website which allows students to simulate the Miller-Urey experiments they are learning about and see the results "first hand". The final link is to a 2011 Science Friday episode talking about the state of the art of this science (first initiated in the 1950's). I provide these links along the lines of the inverted-classroom, a teaching model in which students spend time outside of class mastering course content freeing valuable class time for higher level discussions and other interactive activities. This also allows motivated students can go beyond the material we have time to work with in class.

A#2 Reflection

As new technologies come to the forefront, educators can take advantage of these methods for communicating with students. In addition, modern technologies can also provide teachers and students with novel tools to enrich their learning processes. I appreciate having the ability to give students easy access to supplemental interactive materials that may benefit both struggling students and striving students.

Teaching Large Classes Speaker/Teacher review form: ***Sarah Marsh 4/14/11***

CONTENT:
1) What is the speaker's "5-year" message? What is their main point or essential message? Can you tell easily?
Life is complicated. Yes, well-emphasized.

2) Why is the content/information important? Is the speaker clear about WHY the topic is being presented?
We can learn how to artificially create life. I'm not sure why was emphasized.

3) Is the balance of content and action/engagement done well? Is there a good amount of content being presented in the time allowed? What might help improve this?
I thought there was a good amount of think-pair-share, but between the first video and the next think-pair-share, perhaps more impromptu questions can be used.

DELIVERY:
4) What pedagogical devices are they using? Are they effective?
Blanks in notes for students. I liked the recap summaries. Think-pair-share - asked and then shared after a particular section

Impromptu questions:
5) Are the students around you in class engaged?
Mostly. Other than the two guys in green in the right section

6) Is the speaker loud enough? Is their voice clear?
You were loud and clear, even though I don't think the microphone was working.

7) Are they using the room/space, board, and technology effectively?
Yes, you had a laser pointer and you could move freely around to talk. You also walked around to see how the lecture was doing during think-pair-share.

8) Are their graphics and/or handouts clear and easy to understand?
Yes. I thought more graphics could be used, and perhaps the title could be bold instead. Otherwise, the points were well summarized.

9) Do they move gracefully in and out of activities or questions?
I like it that you walked around to see how the students are doing during discussion.

DEMEANOR
10) Is the speaker enthusiastic? Yes.

11) Is the speaker attentive, reassuring, attuned to the students? Yes.

12) Is the speaker confident and capable in managing the classroom?
Yes. I thought maybe you don't need to be too apologetic about the videos. Also, take note of your space fillers "erm", you use it really often..

13) Does the speaker ASK questions? Does s/he seem interested in the students?
Yes. She questions why students give certain answers.

SUMMARY
What would you say is this speaker's greatest strength?
I like how comfortable you were with interacting with students and I liked the questions you show at the beginning of sections and get them to discuss at the end of a section.

What would most help this speaker improve?
Perhaps just notice that your use of space fillers – "erms"

Other thoughts or comments?
Great job!

ARTIFACT#3 PEER EVALUATION

A#3 Description

This evaluation was written by another student who came to observe me teach as part of an assignment for the Seminar in Teaching Large Classes that I took with Professor Teresa Balser. Throughout my time with Delta, the learning community of other students, staff and faculty who value education has been a critical part of my development as a teacher. This community provided feedback on all levels of my teaching in a safe space. The environment in that seminar as well as other Delta courses has been friendly, open and welcoming. This is one of the best atmospheres to receive criticism to truly improve one's teaching.

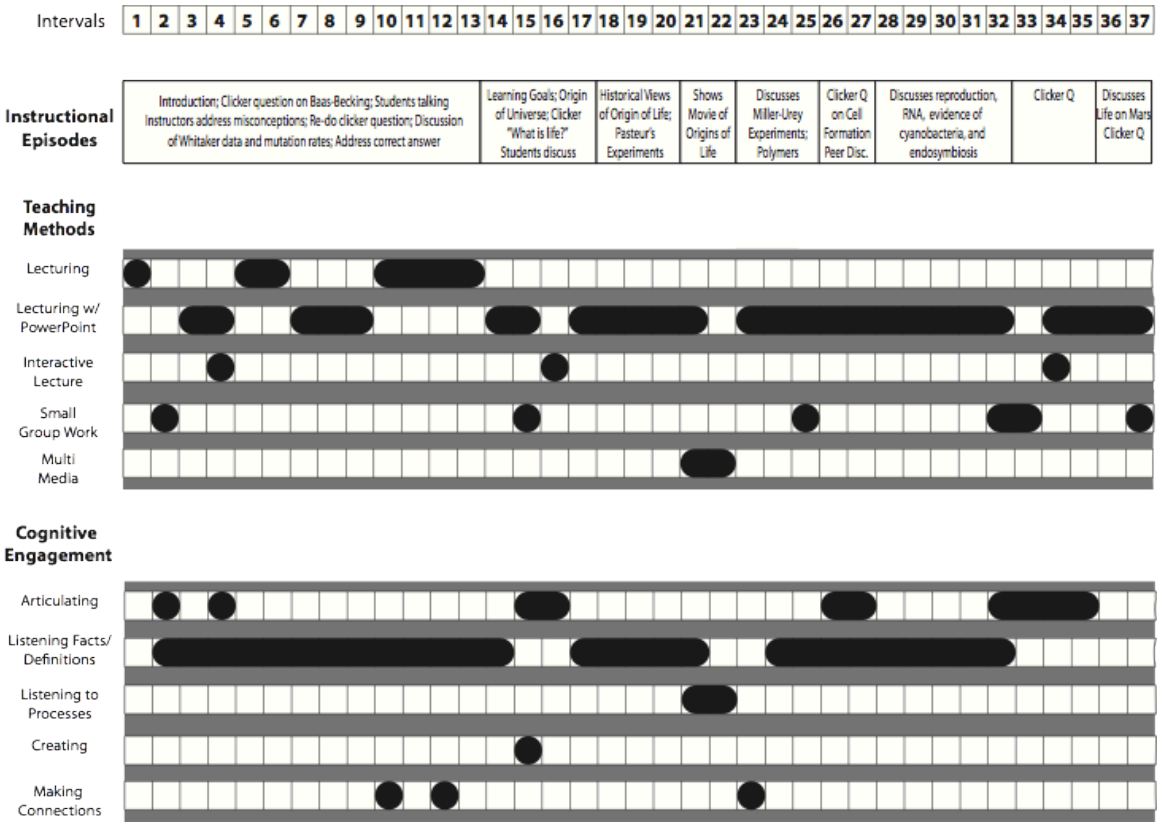
As a part of the seminar, we were encouraged to reflect on what students would ultimately take away from any class we taught. Dr. Balser emphasized the utility of a "sticky" five-year-message. This was both useful to the professor—forcing teachers to hone the essence of their material down to a single important concept, and also to the students—giving them a memorable take-home message. During this process, my classmates gave me feedback on this developing message in-class, and also through an online discussion forum on the Learn@UW website.

A#3 Reflection

Several classmates watched and critiqued my teaching (one evaluation is shown here). Their comments were valuable throughout the process. From the first planning steps through to the conclusion of the classroom teaching with suggestions for how I might improve upon my "finished" product. I gave the "Origin of Life" lecture again the next year so that I could incorporate comments into the material. However, most of the feedback was general enough that

I could start “watching my erms”, as suggested in the summary section of the evaluation, immediately.

Over the course of completing my Delta Certificate I have managed to build an extended network of peers, mainly other graduate students and postdocs, going through the same process. This group shares my passion for teaching and has provided useful feedback and support over the course of the program. Through the Center for the Integration of Research, Teaching and Learning (CIRTL) Network I hope to be able to keep in touch with others sharing this interest as well as maintaining contact with Delta alumni over the course of my academic career.



ARTIFACT#4. EXPERIMENTAL TEACHING OBSERVATION

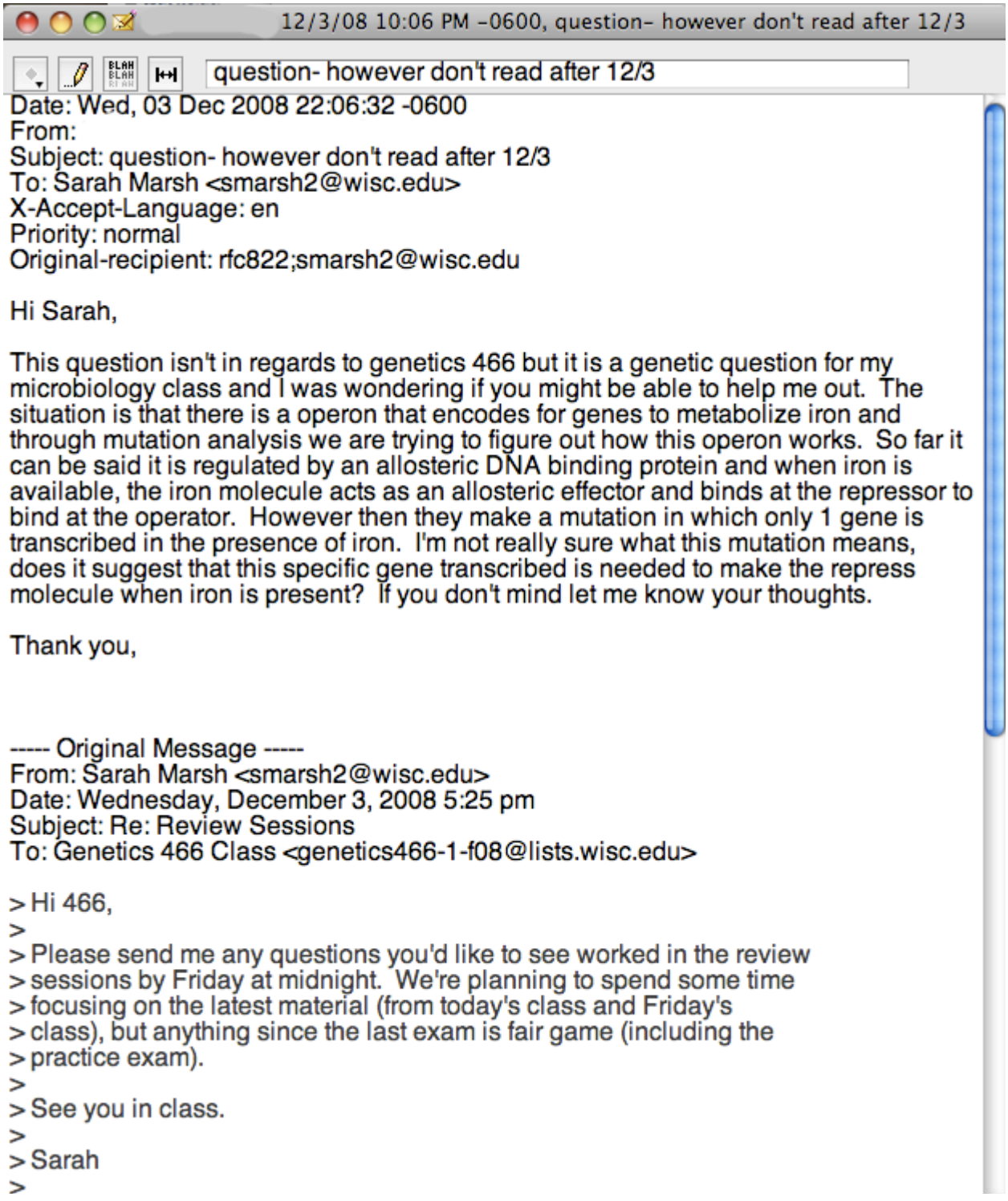
A#4 Description

In the spring of 2012 I participated in a research project designed to provide teachers with improved feedback after classroom observation. The observation protocol was developed by Matthew Hora with the Wisconsin Center for Education Research and was implemented in collaboration with Don Gillian-Daniel, Associate Director of the Delta Program. Dr. Hora observed me teaching the 75-minute Origin of Life lecture discussed in Artifacts 1 and 2, the second year I taught this lecture. He recorded information about my teaching, dividing each observation into two-minute periods. This artifact shows the flow of my lecture. As a part of the study I met with Dr. Hora before the class to discuss my anticipated teaching style and methods. We also met afterwards with Dr. Gillian-Daniel so Dr. Hora could provide feedback on my teaching in different ways and get my comments on which ways of representing the information were most useful.

A#4 Reflection

Classroom observation is a traditional educational practice for K-12 teachers, but much more uncommon at the university level. Since observation tends to be rare, a standard protocol to provide consistent high-quality feedback could be very useful to the educational community. I was eager for additional feedback on my teaching, so volunteered to participate in this study. As all instructors, I was nervous about being observed (although Dr. Hora unobtrusively sat in the back of the classroom and we had talked before the class). I found this objective-style of classroom data collection and feedback useful. One of the ways Dr. Hora quantified my teaching is presented above. This schematic depicts the elements of my teaching and the students' learning. It provided me with independent confirmation of whether I was achieving the

interactive flow I tried to create in class. Participating in this study provided me with some additional feedback on my teaching and helped a future colleague develop what will someday be a widely used educational observation method.



12/3/08 10:06 PM -0600, question- however don't read after 12/3

question- however don't read after 12/3

Date: Wed, 03 Dec 2008 22:06:32 -0600
From:
Subject: question- however don't read after 12/3
To: Sarah Marsh <smarsh2@wisc.edu>
X-Accept-Language: en
Priority: normal
Original-recipient: rfc822;smarsh2@wisc.edu

Hi Sarah,

This question isn't in regards to genetics 466 but it is a genetic question for my microbiology class and I was wondering if you might be able to help me out. The situation is that there is a operon that encodes for genes to metabolize iron and through mutation analysis we are trying to figure out how this operon works. So far it can be said it is regulated by an allosteric DNA binding protein and when iron is available, the iron molecule acts as an allosteric effector and binds at the repressor to bind at the operator. However then they make a mutation in which only 1 gene is transcribed in the presence of iron. I'm not really sure what this mutation means, does it suggest that this specific gene transcribed is needed to make the repress molecule when iron is present? If you don't mind let me know your thoughts.

Thank you,

----- Original Message -----
From: Sarah Marsh <smarsh2@wisc.edu>
Date: Wednesday, December 3, 2008 5:25 pm
Subject: Re: Review Sessions
To: Genetics 466 Class <genetics466-1-f08@lists.wisc.edu>

> Hi 466,
>
> Please send me any questions you'd like to see worked in the review
> sessions by Friday at midnight. We're planning to spend some time
> focusing on the latest material (from today's class and Friday's
> class), but anything since the last exam is fair game (including the
> practice exam).
>
> See you in class.
>
> Sarah
>

ARTIFACT#5 STUDENT EMAIL

A#5 Description

I received the email above while TAing for Genetics 466 with professors Dr. Sean Carroll and Dr. John Doebley. This advanced genetics class includes microbial genetics, however the student's question originated in another course.

A#5 Reflection

At first I was annoyed by this email—wasn't this some other TA's responsibility? After thinking about how I would answer the email, I realized that I should instead be flattered. I had created an environment in which the student felt comfortable to ask questions. Establishing an open intellectual environment was especially important since one of the genetics professors' goals was to teach students to apply the logical problem solving they learned and practiced in genetics to other situations. This kind of flexibility in teaching and applying knowledge across disciplines is valuable in educators at all levels.

ARTIFACT#6 TEACHING-AS-RESEARCH: A DELTA INTERNSHIP REPORT

THE EFFICACY OF USING A CURRENT-EVENTS BASED CASE STUDY TO TEACH THE PRINCIPLE BEHIND EVOLUTION BY NATURAL SELECTION IN MICROBES

Abstract

Many classroom-based activities have been developed to reinforce the major principles behind the mechanisms of evolution by natural selection. However, almost none of these exercises are designed around bacteria or other microbes. I designed a current events-base case study for a Microbial Ecology, Evolution and Diversity course that allowed students to design experiments to test for evolution by natural selection in microbial populations. I implemented the case study as a group assignment working with my internship partner, Dr. Trina McMahon. I gauged the efficacy of this tool using pre- and post-assignment iClicker questions. These questions were designed to test understanding of specific concepts. I found learning gains across all questions, with the greatest improvement for content areas with the lowest initial scores. We consented all willing students and only used data from those present for both the pre- and post- question lectures. Overall student scores significantly increased through case study participation ($p=0.049$, $n=44$). This improvement was also observed in a small number of students working independently ($n=2$) as well as in students who had selected an exam-only grading scheme ($n=3$) who discussed the assignment but did not turn-in the written component. Future work increasing sample sizes for these solo and non-graded groups will be needed to verify these trends, however, current results suggest that case study participation strengthens student understanding of the core mechanisms operating in evolution by natural selection.

Introduction

PROBLEM ADDRESSED

Many classroom-based activities have been developed to reinforce the major principles behind the mechanism of evolution by natural selection. However, almost none of these exercises are designed around bacteria or other microbes. I designed a current events based case study for an upper level Microbial Ecology, Evolution and Diversity course in which students develop tests of evolution by natural selection in microbial populations. This case study focused on the reaction of local microbial populations to the New Horizon oil spill, asking students to assess whether changes in microbial population occurred through evolution by natural selection or other ecological and evolutionary processes.

CURRENT STATE OF THE PROBLEM IN THE LITERATURE

Case studies can be useful in engaging students (Smith & Murphy 1998), particularly when centered on current events (Mysliwiec et al 2003). In the 1990's Herreird (1994) proposed using case studies, then commonly used to teach business, law, and medicine, to teach science. Case studies facilitate active learning, encouraging students to develop critical thinking and teamwork skills. In addition, they appeal to students on a pragmatic level to solve real-world problems, while reinforcing fundamental scientific concepts. There are currently no case studies available to teach the fundamental tenants of evolution by natural selection in bacteria. Numerous studies have shown the benefits of having students work in groups (Michaelsen et al 2002). Jones developed a microbial molecular biology case study centered on gulf oil spill remediation (2011). While this activity's learning goals are quite different than my own, this case study provides a useful example centered on the role of microbes in a salient current event.

TEACHING-AS-RESEARCH QUESTION

Will students' understanding of the principles behind evolution by natural selection improve after participation in a case study requiring them to design experiments to assess whether a microbial population is responding to an environmental challenge by evolving or (physiologically) adapting?

Teaching-As-Research Planned Approach

DESIRED GOALS & OUTCOMES

After completing the case study, I would like students to be able to demonstrate understanding of the principles necessary for evolution by natural selection to occur. I would also like them to be able to apply this knowledge to new problems.

ADDRESSING THE PROBLEM: PLANNED ACTIVITIES

I developed a current-events based case study to be worked in groups that exposed students to a real-world example of principles explained in class (see Appendix 1 for the case study assignment). I selected the New Horizon oil spill example to reinforce the relevance of evolutionary principles, hoping to give this theory modern resonance and applicability. The case study assignment not only presents the material from another viewpoint, but also allows them to think critically and creatively while developing problem-solving skills.

SPECIFIC TEACHING & LEARNING APPROACHES TO ADDRESS A DIVERSE STUDENT BODY

The case-based-learning activity incorporated a variety of specific approaches tailored to facilitate learning among a diverse group of undergraduate students. This activity was done in small groups of three to four so that the students can benefit from discussing the content with one

another. The groups were assigned to maximize gender and ethnic diversity. Having the instructor pre-select groups also ensured that students would work with a mix of people they did not necessarily know well beforehand. This type of group work has been shown to generate more creative solutions and improved student learning (Michaelsen et al 2002, reviewed in Prince 2004) In addition I presented the case study using a variety of modalities—including a written document, lecture with slides, a video and additional resources on the course Moodle website. The students discussed the case study, the majority working with laptops to access a variety of materials including original research articles, coming together to produce a single written document representing the group's effort. This variety in teaching and learning modes was designed to provide the repetition of information all learners need to retain content and present the material in the favored format for many of the students' diverse learning styles.

USE & DEVELOPMENT OF LEARNING COMMUNITIES

Students were given more than 75 minutes of in-class time to work on the case study in groups. The assignment was designed to help promote learning through interactions between students from diverse backgrounds. Small groups of three to four students were designed to enable quiet and shy students to have their voices heard as part of the learning community. The students were able to work together during some class time, but no group was able to complete the entire assignment in class. Many groups also met outside of class. Most students reported spending some time working independently outside of class as well (pers. comm.). We hope that students in need of study groups will continue to work with others from this pre-assigned group over the course of the class.

Evaluation

KEY EVALUATION QUESTIONS

I began developing the case study by thinking about the core ideas I wanted my students to take away; writing paired iClicker questions to assess their understanding of these key areas immediately before and after the activity (see Table 1).

Table 1 iClicker Assessment Questions. Below are the paired pre- and post- assessment iClicker Questions used to evaluate student understanding. Students were told the questions were intended to be straightforward and not an effort to trick them. For example, an entirely phenotypically homogeneous bacterial strain would respond the same way under any culture conditions, even though this might be unlikely to be maintained for long due to high bacterial mutation rates.

Category	Pre-assessment Question	Post-assessment Question
Variance	True/False: Evolution by natural selection could occur in an entirely phenotypically homogenous population of bacteria.	True/False: Evolution by natural selection could occur in an entirely genotypically homogenous population of bacteria.
Inheritance	True/False: Traits unaffected by genetics can evolve by natural selection.	True/False: Non-heritable traits can evolve by natural selection.
Resource limitation	True/False: Bacteria are frequently resource limited in nature.	True/False: While labs commonly use minimal media for bacterial selections, bacteria are rarely resource limited in nature.
Fitness-general	True/False: For evolution by natural selection to occur environmental conditions MUST influence fitness.	True/False: Evolution by natural selection can occur under conditions that do not effect fitness.
Fitness-bacterial	True/False: The bacteria with the fastest doubling time in a population is ALWAYS the most fit.	True/False: The bacteria with the slowest doubling time in a population is ALWAYS the least fit.
Natural selection	True/False: For Evolution by natural selection to occur survival MUST be non-random.	True/False: For Evolution by natural selection to occur survival MUST be random.

ASSESSMENT TECHNIQUES/APPROACHES

Assessment includes both formative as well as summative assessments. I incorporated questions into the course's pre-class evaluation, administered on the first day of class to gauge students' baseline knowledge of and coursework pertaining to evolution. I used the iClicker questions

above to isolate conceptual gains specific to the case study. The pre-questions were administered during the lecture I gave introducing the case study, while the post-questions were given as part of an exam review session on the day the written assignment was due. In addition, the activity itself is designed to provide formative assessment during the learning process. I included questions on the student's midterm exam as well as to evaluate knowledge retention and provide a summative assessment.

Data Analysis & Results

Conceptual Understanding

I compared student scores on paired pre- and post- iClicker questions, as well as the six questions together. These comparisons were statistically significant by a paired two-tailed t-test for three questions (variance p=4.59x10-9, general fitness p=0.025, and natural selection p=0.003) as well as for the pooled sample (n=44, p=0.049) (see Figure 1).

Differences between groups

I used the Krustal-Wallace Rank Sum test (a non-parametric test designed to test for differences between non-normally distributed groups) to assess possible differences between the students who completed the case study (n = 44), those who completed it individually (n=2) and a third group of students in an exam-only grading scheme (n=3) who discussed the case study but did not turn-in a written component. For these comparisons I analyzed improvement in student scores rather than raw scores since the two students completing the case study alone had lower pre-score than the other groups. None of these comparisons were statistically significant, but the

low number of students completing the case study in groups of one and ungraded were not high enough for these tests to have meaningful statistical power (see Figure 2).

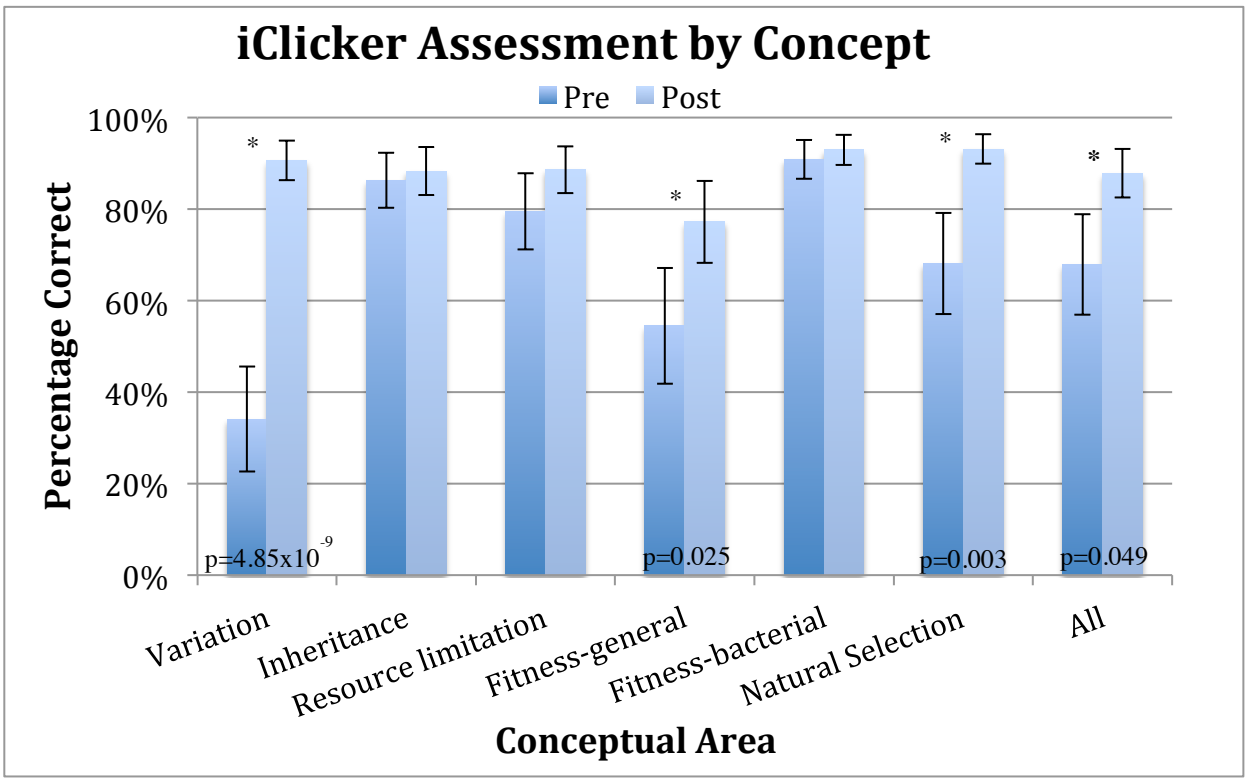


Figure 1. Conceptual Gains by Area. This graph compares the percentage of the students answering iClicker questions on evolution by natural selection before and after the case study. * denote significant differences in pre- and post- scores.

IMPACT OF PRIOR KNOWLEDGE

To assess the possible impact of prior knowledge on students learning gains nor ultimate understanding of the material. I ran linear regressions of pre- assessment scores against the number of prior evolution courses the students self-reported on their initial assessments. I also ran a linear regression of the students' scores against question on the initial evaluation asking them to name and describe at least two of the main principles behind evolution by natural selection. Neither of these regressions revealed a correlation. Prior knowledge did not seem to impact students' learning gains nor ultimate understanding of the material.

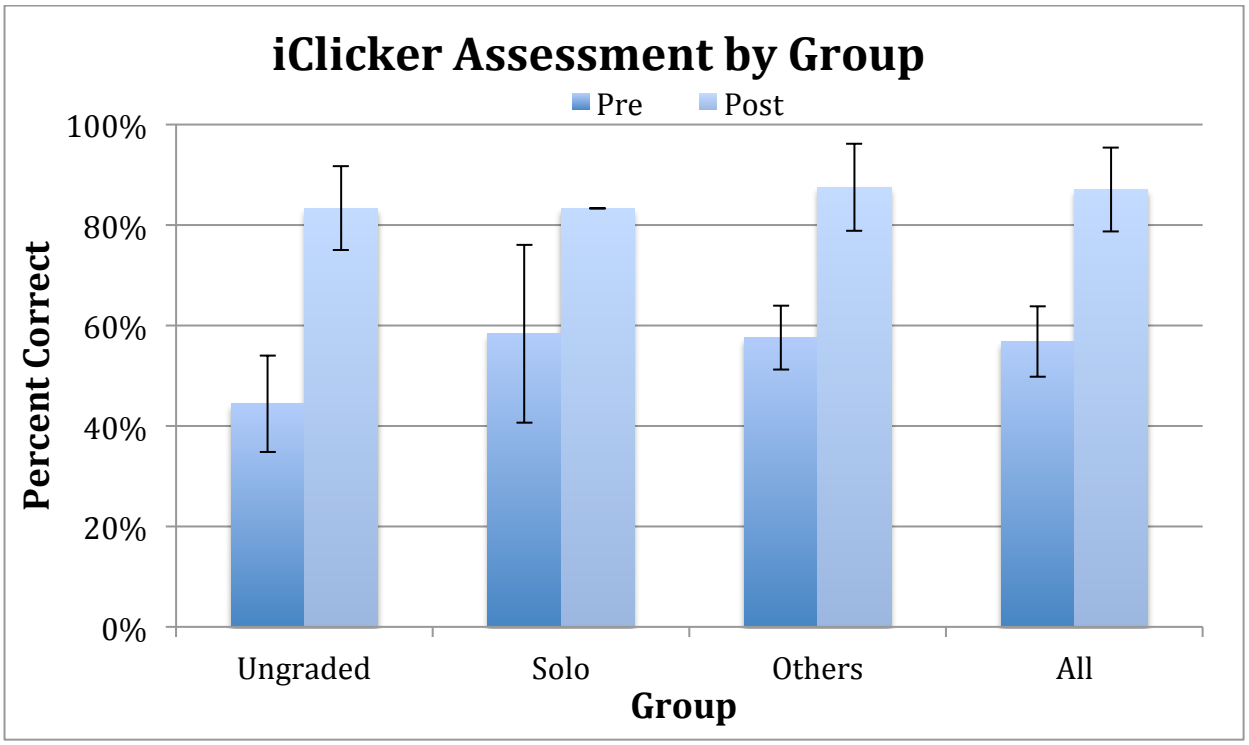


Figure 2. Group learning gain comparison. This graph compares the percentage of correct answers between groups. There were no significant differences between groups.

Long-term Learning Gains

To evaluate how well the students retained these concepts over time, I compared their post-case study assessment scores against their midterm grades. I divided the questions on the midterm into those related and unrelated to evolution. Evolution-related midterm scores correlated with post-assessment scores ($R^2=0.2887$), while unrelated midterm scores showed no relationship to post-assessment scores ($R^2=0.0278$) (see Figure 3). This suggests that learning gains from the case study project were maintained over time.

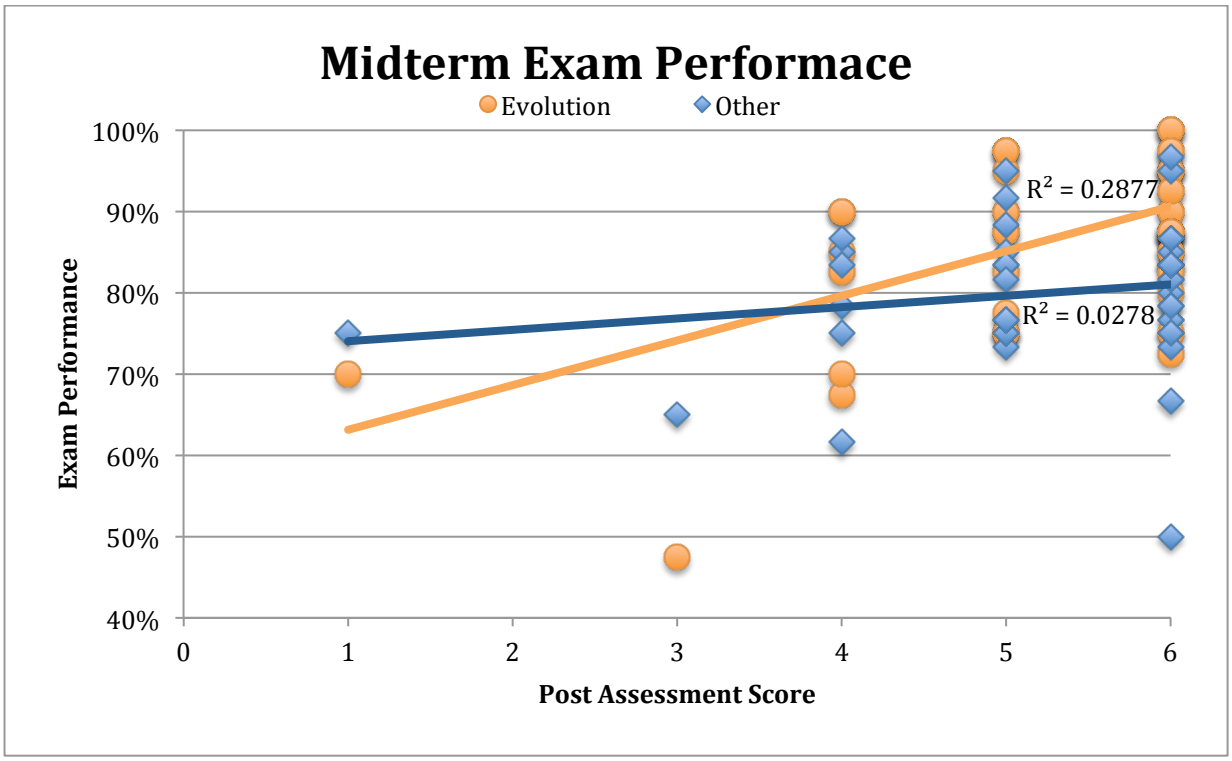


Figure 3. Midterm exam performance. This figure plots students' exam performance against their post-case study iClicker scores. Evolution questions (circles) are related to post-assessment scores (R2=0.2887) while other subject areas (diamonds) are essentially unrelated (R2=0.0278).

Discussion

Summary.

This study designed, implemented and evaluated the efficacy of a current events-based case study teaching evolutionary concepts to upper division microbiology students. The case study challenged students to propose their own experiments to determine whether microbial responses to the 2010 New Horizon oil spill were due primarily to evolution by natural selection. Students completed the assignment in groups to leverage diversity and foster smaller learning communities within the class. Results suggest that case study participation strengthened understanding of the key evolutionary principles. Future work will be needed to distinguish whether completing the written assignment or working in groups were necessary components to

achieve these gains.

INTERPRETATION

These results support the efficacy of the case study model of active learning as previous authors have found (Smith & Murphy 1998, Herreid et al 1994). This activity achieved learning gains while utilizing groups to generate creative experimental solutions as previously demonstrated by Michaelsen and colleagues (2002). This work developed a new case study to teach the fundamental principles of evolution by natural selection using microbial examples, an area which was previously lacking.

APPLICATIONS OF RESULTS

these results suggest that the case study as designed and implement was effective in achieving learning gains in concepts related to evolution by natural selection. These data suggest that using the case study in the future would improve student understanding in this difficult area. The iClicker measurement technique was easily to integrated into an active learning classroom environment, and allowed me to easily collect appropriate data.

LESSONS LEARNED

Student groups had productive discussions as part of the case study process. Based on my limited sample size, group work generated creative problem solving solutions and lead to long-term improvement in students' understanding. Next time the case study is implemented I would add a 5-page limit (1 single-spaced page per principle with an additional page for introductory material, implications, references etc). I would also draw a stronger contrast between ecological and evolutionary processes and incorporate a slide into my introduction giving students tips

structuring group work.

Conclusions

Completing the case study significantly increased student understanding of key concepts of the mechanisms of evolution by natural selection. These gains appear to be reflected in student performance on evolutionary midterm exam questions, suggesting longer-term concept retention from the case study. A similar improvement in pre-/post- scores was also observed in both students working independently and student who did produce a written document. These latter groups were too small (five students total) for a statistically meaningful comparison. Future studies with larger sample sizes in these categories will be needed to evaluate possible trends in these areas.

Bibliography

Culp T. 1999. Demonstrating Natural Selection Using Magnetobacteria. Am Biol Teach. 61: 616-620.

Delpech R. 2009. Evolution in an Afternoon: Rapid Natural Selection and Adaptation of Bacterial Populations. J Biol Educ 43: 129-133.

Herreid CF. 1994. Case studies in science—A novel method of science education. J Coll Sci Teach. 23: 221–229.

Kagle J & AG Hay. 2007. Microbial Evolution is in the Cards: Horizontal Gene Transfer in the Classroom. Am Biol Teach. 69: 23-28.

Krist AC & SA Showsh. 2007. Experimental Evolution of Antibiotic Resistance in Bacteria. Am Biol Teach. 69: 94-97.

Jones DR. 2011. Case Study: Using Microbe Molecular Biology for Gulf Oil Spill Clean Up. Biochem Mol Biol Educ. 39: 157-164.

Michaelsen L, Knight A & L Fink, Eds. 2002. Team-Based Learning: A Transformative Use of Small Groups. Westport CT: Praeger Publishers.

Mysliwiec T, Shibley I & M Dunbar. 2003. Using newspapers to facilitate learning: Learning activities designed to include current events. J Coll Sci Teach. 33: 24–28.

Prince M. 2004. Does Active Learning Work? A Review of the Research. J Engr Education. 93: 223-231.

Serafini A & DM Matthews. 2009. Microbial Resistance to Triclosan: A Case Study in Natural Selection. Am Biol Teach. 71: 536-540.

Smith RA & SK Murphy. 1998. Using case studies to increase learning and interest in biology. Am Biol Teach. 60: 265–268.

Stromberger ME. 2005. Fire Vs. Metal: A Laboratory Study Demonstrating Microbial Responses to Soil Disturbances. J Natur Resour Life Sci Educ. 34:1-7.

Appendices

APPENDIX 1.1 CASE STUDY IN MICROBIAL EVOLUTION: NATURAL SELECTION & THE DEEPWATER HORIZON OIL SPILL

A case study in bacterial evolution:
Natural selection & the Deepwater Horizon oil spill

Assignment: Work in assigned groups of 3-4 students to develop experiments designed to test whether evolution by natural selection is occurring in response to the Deepwater Horizon oil spill. Sarah Marsh, a teaching intern, will introduce the project and you will have time to meet in your groups briefly. You'll also have the entire class time Thursday, March 15th to work on this. Sarah will also be at Gina's office hours on Monday, March 19th if you have any questions after class-time. Each group should submit a typewritten document through the Moodle site DUE BEFORE CLASS STARTS TUESDAY MARCH 20TH 2012 including the following sections:

Project Participants: A list of the students in your group.

Experimental Design & Projected Results:
Overview. Begin this section describing the big-picture question you are testing. Include a description of the environment you selected for your experiments. Be sure to justify your choice, explaining why this is a good choice—you might consider ease of access, cost, and significance of the data you will collect.

[The subsections below should be repeated at least four times—once for each major principle.]
Experiments.
How would you test whether this principle is at work here? Brainstorm potential hypotheses and methods in your group. Describe the experiments you would use in this situation— what kind of data will this generate? How will you analyze it?
Justification.
Justify the method you chose for your assessment—why is it better than alternative possibilities (think about cost, ease and impact)?
Controls.
What factors do you need to control? How will you do this?
Replication.
What is your sampling scheme? How many biological replicates (designed to test the bacteria themselves) will you collect? Do you need technical replicates (designed to test the accuracy and precision of the measurements themselves)?
Hypotheses.
Include a hypothesis and at least one alternative hypothesis.
Expected results.
Describe what you would expect to see and why.

Implications: A paragraph discussing the potential implications your experiments might have for oil spill remediation both for this event and future spills.

References: Bibliography information for sources you may have cited. Feel free to use the references below or to do additional outside research if you like. This is not necessary to do earn

a high grade on the assignment. Be sure to keep source quality in mind source quality—journal articles are much higher quality than Wikipedia. Your grade will reflect this.

Roles & Effort: Each person should turn in separate sheet describing their own and the other team members' contributions to the project and gauging their percent effort. This section is to keep everyone honest. It will count towards your professional conduct grade.

Background

Oil spill information:

In April 2010 the Deepwater Horizon oilrig exploded and sank, resulting in the largest oil spill in US waters on record. Nearly five million gallons of oil and natural gas were released into the Gulf of Mexico. Methane made up approximately 20% of the hydrocarbons released. Four months later methanotrophs, undetectable at the time of the spill, were common in the water column. Over this period methane had dropped to undetectable levels (Kessler et al 2011). However, much of the rest of the oil has either reached the shoreline or sank to the ocean floor where it can remain for decades. Changes in microbial communities associated with the oil spill were apparent in the water column itself (Hazen et al 2010, Gauglitz et al 2012, Lu et al 2012, Valentine et al 2012), and on shore (Kostka et al 2011). Human pathogens have even been documented at high levels in tar balls associated with the spill (Tao et al. 2011).

Major principles behind the mechanisms of evolution by natural selection:

Individuals in a population vary (are not phenotypically/genetically identical)
This variation is heritable
Individuals vary in their reproductive success (fitness)
Keep in mind that resource limitation prevents populations from expanding indefinitely
Variation is non-random (natural selection, fitness depends on the environment)

Discuss these questions in your group to help develop experiments:*

Explain why variance in the population is necessary for natural selection to act.
Does all the variation in a population need to be heritable? Justify your answer.
In this situation, list the resources you think are limiting and explain why.
Define fitness. Explain why fitness might vary in different environments.
Define natural selection. What selective pressures are at work in this situation?

* You do not need to turn in written answers to these questions, but talking about them may help you if you're stuck designing experiments.

Problem/Procedure: Your main goal is to determine whether potential changes in the populations you observe are due to evolution by natural selection. You will be designing an experiment to test each of the four principles discussed above. You are in the same position as researchers when the oil spill occurred—that is you can "time-travel" back to the time of the spill, but not beforehand. First decide where you will focus your study—in the water column, on-shore, or on the deep ocean floor. Think about what types of samples will you collect, the

collection time course and the types of data will you generate from these samples. (See below. This list is neither exhaustive, nor do you have to use all the methods on it to earn full credit.)

Possible data types:
Oceanographic data including:
location, temperature, depth, salinity, dissolved oxygen & hydrocarbon content
Culturing data including experiments with selective media and passaging
Pyrosequencing 16S ribosomal DNA libraries
Surveys DNA present in the environment
Meta-genomics
Measures comparative quantities of DNA or RNA present in the environment
Quantitative real time polymerase chain reaction (qRT-PCR)
Amplifies and measures DNA or RNA in the environment

References

Gauglitz JM, Zhou H & A Butler. 2012. "A suite of citrate-derived siderophores from a marine Vibrio species isolated following the Deepwater Horizon oil spill." J Inorg Biochem. 107: 90-95.

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'haeseleer P, Holman HY, Osman S, Lu Z, Van Nostrand JD, Deng Y, Zhou J & OU Mason. 2010. "Deep-sea oil plume enriches indigenous oil-degrading bacteria." Science. 330: 204-208.

Kessler JD, Valentine DL, Redmond MC, Du M, Chan EW, Mendes SD, Quiroz EW, Villanueva CJ, Shusta SS, Werra LM, Yvon-Lewis SA & TC Weber. 2011. "A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico." Science 331: 312-315.

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC & M Huettel. 2011. "Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill." Appl Environ Microbiol. 77: 7962-7974.

Lu Z, Deng Y, Van Nostrand JD, He Z, Voordeckers J, Zhou A, Lee YJ, Mason OU, Dubinsky EA, Chavarria KL, Tom LM, Fortney JL, Lamendella R, Jansson JK, D'haeseleer P, Hazen TC & J Zhou. 2012. "Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume." ISME J. 6: 451-460.

Tao Z, Bullard S & C Arias. 2011. "High Numbers of Vibrio vulnificus in Tar Balls Collected from Oiled Areas of the North-Central Gulf of Mexico Following the 2010 BP Deepwater Horizon Oil Spill." Ecohealth. Epub ahead of print.

Valentine DL, Mezic I, Macesic S, Crnjaric-Zic N, Ivic S, Hogan PJ, Fonoberov VA & S Loire. 2012. Dynamic autoinoculation and the microbial ecology of a deep water hydrocarbon irruption. Proc Natl Acad Sci USA. Epub ahead of print.

Example Document

Project Participants: Gina Lewin, Sarah Marsh, Trina McMahon & Streptomyces bikiniensis

Experimental Design & Projected Results:
Overview. We propose to test whether more students attend if they can chew gum during class. We will sample students in Micro450 because we will already be there to attend class helping to minimize data collection costs and time. In addition, the class is sufficiently large so we expect to be able to detect statistically significant trends. This work will have significant implications for classroom attendance as well as university conduct rules.

Part 1.
Experiments. To test whether more students are attending class we will count the number of students in class before and after the rule change. In an effort to tease apart whether this change can be attributed to gum chewing we will also count the number of students chewing gum at the beginning, middle and end of class.
Justification. We chose counting because this method is highly cost-efficient, easy to replicate and requires no special equipment in contrast to student surveys that require distribution, student effort and could be biased by memory.
Controls. To control for attendance changes due to weather and stress, we will analyze the percentage of class chewing gum on any particular day. In addition, we will monitor the students' desks for food to control for snacking. We will also count the number of hair chewers and fingernail biters since these habits are difficult to break and there will be no rule change regarding these areas so we would not expect their frequency to change.
Replication. We will sample the entire class. In this experiment each student represents a biological replicate. We will monitor the class on three days before the rule change and three days after (technical replicates).
Hypotheses.
Null hypothesis: There will be no change in the number of students chewing gum.
Alternative hypothesis 1: More students will chew gum.
Alternative hypothesis 2: Fewer students will chew gum.
Expected results. More students will attend class if gum chewing is allowed because students like to chew gum.

[Parts 2-4.]

Implications: Our results suggest that professors should allow gum chewing in class to improve attendance. We believe that this may be due to students' preference for a relaxed classroom environment. It might also be because of an increase in good breath among classmates. To further test this, we propose additional studies comparing gum only, breath mint only, gum and breath mint friendly and restricted classrooms. For now, we recommend that the University of Wisconsin allow gum chewing to increase class attendance regardless of the mechanism at work.

References: See above

Roles & Effort: (Sarah's estimates, each person submits a separate document for this)
Gina—Brainstormed hypotheses, drafted section Parts 1 and 2, edited others; (100%)
Sarah—Brainstormed hypotheses, researched references, drafted Part 3, edited (100%)
Trina—Brainstormed hypotheses, designed experiments, drafted Part 4, edited others (100%)
Streptomyces bikiniensis—Didn't really contribute, more of a parasite on the group (20%)

APPENDIX 1.2. RUBRIC

Rubric: Out of 100 (105 possible)

Project Participants: +5
Full credit for including section with first and last names
Reduced credit if first or last names only
No credit if section missing

Experimental Design & Projected Results: +80 (20 points per principle)
Hypothesis: 5
Experimental design: 15
Justification: 5
Controls: 5
Expected results: 5

Implications: +10
Full credit for attempting to apply your conclusions to future oil spills in a logical way
Reduced credit for logical flaws

References: +5 (optional section)
Full credit for properly cited primary reference(s)
Partial credit for secondary sources (wikipedia etc)
No credit if none listed

Roles & Effort: +10
Full credit for 100% effort
Reduced credit for lessor effort

Reflection

Delta Internship Teaching-as-Research Project. I completed my Delta internship in the spring of 2012. I developed and implemented a case study designed to teach the principles behind evolution by natural selection in a Microbial Ecology, Evolution and Diversity course for upper division students. This was my first Teaching-as-Research endeavor so many of the lessons were hands-on. The internship also gave me the opportunity to implement group teaching and learning strategies to take advantage of Learning-through-Diversity and to create smaller Learning Communities within the larger class cohort.

Teaching-as-Research. The primary goal of my Delta internship project was to improve student understanding of the principles behind evolution by natural selection, an area in which students have traditionally performed poorly in the past. I designed a case study in which the students worked in teams discussing key concepts and designing experiments around them. However, I wanted to be able to assess student learning individually. To do this I wrote paired pre- and post-iClicker questions covering each of four major concept areas. This allowed me to assess both individual and aggregate gains, either independently from or in tandem with their other learning indicators including pre-course assessment, case study and exam scores. The pre- questions were essential to establish a baseline since the concepts I emphasized were also taught in the course prior to the case study assignment. Using iClicker questions enabled me to perform targeted as well as aggregate analyses. It also provided useful formative feedback during the class sessions. I plan to continue using these type of questions as well as other formative assessment methods in the future for teaching-as-research in day-to-day activities as well as larger projects for publication.

Learning Community. I fostered learning communities for my students while at the same time benefiting from my own internship-class learning community. I worked with a group of approximately 70 students, assigning them to groups of three to four students to create smaller learning communities. The case study included several suggested questions to get groups comfortable talking before they tackled the main objectives of the assignment. I gave the students more than 75 minutes to work on the case study together in class. This format established an atmosphere in which the students could begin project discussion with easy access to teacher support for questions. In addition, this time allowed the groups to schedule additional outside of class meetings if needed.

On my side, the Delta internship cohort was a supportive learning community. Their feedback was extremely valuable. It was also reassuring to hear others talk about problems they faced in their projects similar to the ones I was experiencing. Connection with other learners is an important part of the educational process at all levels.

Learning-through-Diversity. I used group-work to take advantage of the diversity in my student population. The students I taught were mainly microbiology majors. However, the course included some students from other departments, and every student was likely to come with a unique variety of research experiences. I assigned case-study groups in an effort to maximize diversity in gender and ethnicity as well as to encourage students to work with others they might not know well. Students in the majority of these groups appeared to discuss the assignment freely and interact well.

Synthesis. My Delta internship was a capstone experience during which I implemented a Teaching-as-Research project. This active learning helped to solidify my understanding of the Teaching-as-Research process. I was also able to take advantage of my own Delta cohort as a learning community while at the same time cultivating learning communities for my student through group work. Assigning groups also helped me to tap into the diversity inherent within students in all courses by mixing students across ethnicity, gender and experience. I plan to incorporate these principles into my teaching toolkit both for future courses and other learning relationships.

APPENDIX 2. THE GENOME SEQUENCE OF THE LEAF-CUTTER ANT ATTA CEPHALOTES REVEALS INSIGHTS INTO ITS OBLIGATE SYMBIOTIC LIFECYCLE

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Author contributions: Conceived and designed the experiments: GS LL CT TTH JT SCS SWC WCW CGE GMW NMG CRC. Performed the experiments: CT LL PB EJC SEM WCW. Analyzed the data: GS LL CH EA EBB EJC EC AC EE HH MJF JG JDG DG KJG DEH MH CH HH BRJ JK SEM JAM MCM MCMT MCN SN RO RR JTR CRS ST NDT LW MDY LV FZ CGE CDS NMG CRC. Contributed eagents/materials/analysis tools: CT LL CH PB HH JJS MDY TTH SWC WCW CGE CDS GMW. Wrote the paper: GS. Project Management: GS CT TTH JT SCS SWC CGE GMW NMG CRC. (SEM isolated all DNA and RNA for the genome and annotated a portion of the olfactory genes

I. ABSTRACT

Leaf-cutter ants are one of the most important herbivorous insects in the Neotropics, harvesting vast quantities of fresh leaf material. The ants use leaves to cultivate a fungus that serves as the colony's primary food source. This obligate ant-fungus mutualism is one of the few occurrences of farming by non-humans and likely facilitated the formation of their massive colonies. Mature leaf-cutter ant colonies contain millions of workers ranging in size from small garden tenders to large soldiers, resulting in one of the most complex polymorphic caste systems within ants. To begin uncovering the genomic underpinnings of this system, we sequenced the genome of *Atta cephalotes* using 454 pyrosequencing. One prediction from this ant's lifestyle is that it has undergone genetic modifications that reflect its obligate dependence on the fungus for nutrients.

Analysis of this genome sequence is consistent with this hypothesis, as we find evidence for reductions in genes related to nutrient acquisition. These include extensive reductions in serine proteases (which are likely unnecessary because proteolysis is not a primary mechanism used to process nutrients obtained from the fungus), a loss of genes involved in arginine biosynthesis (suggesting that this amino acid is obtained from the fungus), and the absence of a hexamerin (which sequesters amino acids during larval development in other insects). Following recent reports of genome sequences from other insects that engage in symbioses with beneficial microbes, the *A. cephalotes* genome provides new insights into the symbiotic lifestyle of this ant and advances our understanding of host–microbe symbioses.

II. AUTHOR SUMMARY

Leaf-cutter ant workers forage for and cut leaves that they use to support the growth of a specialized fungus, which serves as the colony's primary food source. The ability of these ants to grow their own food likely facilitated their emergence as one of the most dominant herbivores in New World tropical ecosystems, where leaf-cutter ants harvest more plant biomass than any other herbivore species. These ants have also evolved one of the most complex forms of division of labor, with colonies composed of different-sized workers specialized for different tasks.

To gain insight into the biology of these ants, we sequenced the first genome of a leaf-cutter ant, *Atta cephalotes*. Our analysis of this genome reveals characteristics reflecting the obligate nutritional dependency of these ants on their fungus. These findings represent the first genetic evidence of a reduced capacity for nutrient acquisition in leaf-cutter ants, which is likely

compensated for by their fungal symbiont. These findings parallel other nutritional host–microbe symbioses, suggesting convergent genomic modifications in these types of associations.

III. Introduction

Ants are one of the most successful insects on earth, comprising up to 20% of all terrestrial animal biomass and at least 25% of the entire animal biomass in the New World Tropics [1]. One of the most conspicuous and prolific Neotropical ants are the leaf-cutters (Tribe: Attini), so-called because of their leaf-cutting behavior [2]. Leaf-cutters are unique among ants because they obligately farm a specialized, mutualistic fungus that serves as their primary food source [3]. Using a complex system of trails, foraging ants seek out and cut leaves (Figure 1A) that they use to manure a fungal crop in specialized subterranean fungus gardens (Figure 1B) within their colonies. Fungus farming by ants is exclusive to the New World and is thought to have evolved once 50 million years ago [4], culminating in the leaf-cutter ants. A single mature colony of the genus *Atta* can fill a volume of up to 600 m3 and their fungus gardens can support millions of workers capable of harvesting over 400 kg of leaf material (dry weight) annually [1]. These ants are thus one of the most widespread and important polyphagous insect herbivores in the Neotropics. The importance of leaf-cutter ants in Neotropical rainforest ecology lies in their ability to substantially alter arboreal foliage through their extensive leaf-cutting activities. Estimates suggest that leaf-cutter ants remove 12–17% of the total leaf production in tropical rainforests [1]. As a group, they harvest more plant biomass than any other Neotropical herbivore including mammals and other insects. As a result, leaf-cutter ants are a major human agricultural pest, responsible for billions of dollars in economic loss each year [5]. These ants do, however, have a positive impact on rainforest ecosystems, as they contribute to rapid soil turnover through

their nest excavation activities [6], stimulate plant growth by cutting vegetation [7], and help to recycle organic carbon [1].

In addition to their importance in Neotropical ecosystems, leafcutter ants also serve as a model for understanding the ecology and evolution of host-microbe symbioses [8]. In return for receiving a continuous supply of leaf-material, protection from competitors, and dispersal, the fungus these ants grow provide nutrients in the form of specialized hyphal swellings called gongylidia. Gongylidia, which contain a mixture of carbohydrates, amino acids, proteins, lipids, and vitamins [9], is the sole food source for developing larvae. The fungus garden is also known to harbor other microbial symbionts including nitrogen-fixing bacteria that provide both fungus and ants with nitrogen [10], and a diverse community of fungus garden bacteria that appear to help the fungus degrade plant biomass [11]. The complexity of the leaf-cutter ant symbiosis is further highlighted by the presence of a specialized microfungal pathogen that exploits the ant-fungus mutualism [12,13]. As a result, the leaf-cutter ant symbiosis comprises at least three established mutualists and one specialized pathogen. With the reported presence of additional microbial symbionts from *Acromyrmex* leaf-cutter ants [14–19], and the isolation of numerous microbes from other fungus-growing ants [20–22], this antmicrobe symbiosis is perhaps one of the most complex examples of symbiosis currently described.

Leaf-cutter ants in the genus *Atta* are also known for their morphologically diverse caste system (Figure 1C), which reflects their complex division of labor [23,24]. For example, the overall body size of *Atta cephalotes* workers varies tremendously (i.e., head widths (HW) ranging from 0.6 mm to 4.5 mm [23]), and these differences correspond to the tasks performed by workers.

The smallest workers (HW 0.8–1.6 mm) engage in gardening and brood care as their small mandibles allow them to manage the delicate fungal hyphae and manipulate developing larvae. Some of these workers are also responsible for processing plant material collected by foragers by clipping large pieces of leaf material into smaller fragments to manure the fungus. Larger workers (HW .1.6 mm) are responsible for foraging, as they have mandibles powerful enough to cut through leaves and other vegetation [24]. The largest workers form a true soldier caste, which are involved primarily in nest excavation and colony defense [23,24]. To gain a better understanding of the biology of leaf-cutter ants, we sequenced the genome of *Atta cephalotes* using 454 pyrosequencing technology [25] and generated a high-quality *de novo* assembly and annotation. Analysis of this genome sequence reveals a loss of genes associated with nutrient acquisition and amino acid biosynthesis. These genes appear to be no longer required because the fungus may provide these nutrients. With the recent reports of genomes from other social hymenopterans [26,27] and insects that engage in microbial mutualisms [28,29], the *A. cephalotes* genome contributes to our understanding of social insect biology and provides insights into the interactions of host-microbe symbioses.

I. Results/Discussion

Sequencing, Assembly, and Annotation of the *Atta cephalotes* Genome

Three males from a mature Atta cephalotes colony in Gamboa, Panama were collected and sequenced using 454-based pyrosequencing [25] with both fragment and paired-end sequencing approaches. A total of 12 whole-genome shotgun fragment runs were performed using the 454 FLX Titanium platform in addition to two sequencing runs of an 8 kbp insert paired-end library, and one run of a 20 kbp insert paired-end library. Assembly of these data resulted in a genome

sequence of 290 Mbp, similar to the 300 Mbp genome size previously estimated for A. cephalotes [30]. The genome is spread across 42,754 contigs with an average length of 6,788 bp and an N50 of 14,240 bp (Table 1). Paired-end sequencing (8 kbp and 20 kbp inserts) generated 2,835 scaffolds covering 317 Mbp with an N50 scaffold size of 5,154,504 bp. The disparity between contig and scaffold size may be accounted for by the number of repeats present in this genome (see below) leading to an inflated assembly size due to chimeric contigs. Based on the total amount of base pairs generated and its predicted genome size, we estimate that the coverage of the *A. cephalotes* genome is 18-20X.

To determine the completeness of the *A. cephalotes* genome sequence, we performed three analyses. First, we compared the *A. cephalotes* genome annotation against a set of core eukaryotic genes using CEGMA [31], and found that 234 out of 248 core proteins (94%) were present and complete, while 243 (98%) were present and partially represented. Second, we analyzed the cytoplasmic ribosomal proteins (CRPs) in the A. cephalotes genome and identified a total of 89 genes (Text S1). These encode the full complement of 79 CRPs known to exist in animals, nine of which are represented by gene duplicates (RpL11, RpL14, RpS2, RpS3, RpS7, RpS13, RpS19, RpS28) or triplicates (RpL22). The presence of a complete set of these numerous genes, which are widely distributed throughout the genome, confirmed the high quality of the *A. cephalotes* genome sequence (Text S2). Finally, we found that the genome of *A. cephalotes* contains 66 of the 67 known oxidative phosphorylation (OXPHOS) nuclear genes in insects (Text S3). The only OXPHOS gene missing, cox7a, we found to also be missing in the two ants *Camponotus floridanus* and *Harpegnathos saltator* and the honey bee *Apis mellifera*. The presence of this gene in the jewel wasp *Nasonia vitripennis* (along with other holometabolous

insects), suggests an aculeate Hymenopteraspecific loss, rather than a lack of genome coverage for *A. cephalotes*.

We also generated an annotation for the *A. cephalotes* genome using a combined approach of electronically-generated annotations followed by manual review and curation of a subset of gene models. Expressed Sequence Tags (ESTs) generated from a pool of workers consisting of different ages and castes from a laboratory maintained colony of A. cephalotes was used in conjunction with the MAKER [32] automated annotation pipeline to generate an initial genome annotation. This electronically-generated annotation set (OGS1.1) contained a total of 18,153 gene models encoding 18,177 transcripts (See Materials and Methods), 7,002 of which had EST splice site confirmation and 7,224 had at least partial EST overlap. The MAKER-produced gene annotations were used for further downstream review and manual curation of over 500 genes across 16 gene categories (Table S1). Significant findings from this annotation are highlighted below, with additional details of our full analysis described in Text S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20).

In addition to the *A. cephalotes* genome sequence, we also recovered an 18-20X coverage complete and circular mitochondrial genome, which showed strong whole sequence identity to the mitochondrial genome sequence reported for the solitary wasp *Diadegma semiclausum* [33]. A synteny analysis of the predicted genes on the A. cephalotes mitochondrial genome showed near-identical gene order with that of *A. mellifera* [34] (Text S4).

Repetitive DNA

The A. cephalotes assembly contains 80 Mbp of repetitive elements, which accounts for 25% of the predicted assembly (Table S2). The large majority of these are interspersed repeats, which account for 70 Mbp (21%). Many of these repeats are transposable elements (TEs), with DNA TEs the most abundant and accounting for 14.3 Mbp (4.5%). A large number of retroid element fragments were also identified, with Gypsy/DIRS1 and L2/CR1/Rex as the most abundant. However, the majority of interspersed elements (51.8 Mbp) were similar to *de novo* predictions that we could not be classified to a specific family (Table S2). Improvements to the assembly, integration of repeat annotation evidence, and manual curation will be necessary to determine if these elements represent new TE families or complex nests of interspersed repeats.

Given the obligate association between *A. cephalotes* and its fungal cultivar, we investigated the possibility that the *A. cephalotes* genome might contain transposable elements commonly found in fungi. This was done by re-analyzing the genome using a TE library optimized for the detection of Fungi and Viridiplantae. We did not find evidence for any high-scoring or full-length retroid or DNA TEs from either of these taxa present in the *A. cephalotes* genome.

Our estimate that 25% of A. cephalotes assembly containsrepetitive elements may be ambiguous because our assembly spans 317 Mbp and the estimated genome size for *A. cephalotes* is 300 Mbp [30]. These predictions are, however, more similar to other ant species [27] and *N. vitripennis* [35] than to *A. mellifera* [28], which lacks the majority of retroid elements and other transposable elements (TE) found in *A. cephalotes*.

Global Compositional Analysis

Eukaryotic genomes can be understood from the perspective of their nucleotide topography, particularly with respect to their GC content. Previous work has shown that animal genomes are not uniform, but are composed of compositional domains including homogeneous and nonhomogeneous stretches of DNA with varying GC composition [36]. A global composition analysis was performed for *A. cephalotes* and the compositional distribution was compared to those of other insect genomes, as described in Text S5. This analysis revealed that A. cephalotes has a compositional distribution similar to other animal genomes, with an abundance of short domain sequences and few long domain sequences. *A. cephalotes* also has the largest number of long GC-rich domain sequences when compared to other insect genomes, with over six times the number of long GC-rich domain sequences than the *N. vitripennis* genome. When genes are mapped to compositional domains in the A. cephalotes genome, we find that they are uniformly distributed across the entire genome, in contrast to *N. vitripennis* and *A. mellifera*, which have genes occurring in more GC-poor regions of their genomes.

DNA Methylation

The methylation of genes has been reported for other hymenopterans including *A. mellifera* [37] and *N. vitripennis* [35]. In insects, it is thought that this process contributes to gene silencing [37], but recent reports suggest a positive correlation between DNA methylation and gene expression [38,39]. DNA methylation is thought to involve three genes: dnmt1, dnmt2, and dnmt3 [40], although the precise role of dnmt2 remains unresolved. We found all three genes as single copies in *A. cephalotes*, which is similar to the other ants [27] but in contrast to *A. mellifera* and *N. vitripennis* where dnmt1 has expanded to two and three copies, respectively [35]

(Text S6). Dnmt3 is known to be involved in caste development in *A. mellifera* [41], and the presence of this gene in *A. cephalotes* may therefore indicate a similar role.

RNAi

RNA interference is a mechanism through which the expression of RNA transcripts is modulated [42]. We annotated a total of 29 different RNAi-related genes in A. cephalotes, including most of the genes involved in the microRNA pathway, the small interfering RNA pathway, and the piwi-interacting RNA pathway (Text S7). All detected RNAi genes were found as single copies except for two copies of the gene loquacious. One of these contains three double-stranded RNA binding domains characteristic of loquacious in D. melanogaster [43], whereas the other contains only two. It is not known what role this second loquacious-like gene plays in *A. cephalotes* and future work is needed to deduce its role.

The Insulin Signaling Pathway

The insulin signaling pathway is a highly-conserved system in insects that plays a key role in many processes including metabolism, reproduction, growth, and aging [44]. An analysis of the insulin signaling system in A. cephalotes reveals that it has all of the core genes known to participate in this pathway (Text S8). One of the hallmarks of A. cephalotes biology is its complex sizebased caste system and, although virtually nothing is known about the genetic basis of caste development in this ant, it is currently thought that it is intrinsically linked to brood care and the amount of nutrients fed to developing larvae [1]. Given the importance of the insulin signaling system in nutrition, it is likely that this pathway is involved in caste differentiation in *A. cephalotes*, as has been shown for *A. mellifera* [45].

Yellow and Major Royal Jelly Proteins

The yellow/major royal jelly proteins are encoded by an important class of genes and in *A. mellifera* they are thought to be integral to many major aspects of eusocial behavior [46]. For example, members of these genes are implicated in both caste development and sex determination. An analysis of this gene family in *A. cephalotes* revealed a total of 21 genes, 13 of which belong to the yellow genes and 8 of which encode major royal jelly proteins (MRJP) (Text S9). In general, the yellow genes display one-to-one orthology with yellow genes in other insects like *Drosophila melanogaster* and *N. vitripennis*. With eight members in the MRJP subfamily, which is restricted to Hymenoptera, the number of MRJP genes in *A. cephalotes* is similar to the number reported for other Hymenoptera [35,46]. However, five of the eight genes in *A. cephalotes* are putative pseudogenes. This may indicate that a high copy number of MRJPs may be an ancestral feature and that *Atta* is in the process of losing these genes. The loss of MRJPs may be a common theme among ants, as the recently reported genome sequences for *C. floridanus* and *H. saltator* revealed only one and two MRJP genes, respectively [27].

Wing Polymorphism

Wing polyphenism is a universal feature of ants that has contributed to their evolutionary success [1]. The gene networkthat underlies wing polyphenism in ants responds to environmental cues such that this network is normally expressed in winged queens and males, but is interrupted at specific points in wingless workers [47]. We therefore predict that the differential expression of this network between queens and workers may be regulated by epigenetic mechanisms as has been demonstrated in honey bees [41]. In A. mellifera, developmental and caste specific genes have a distinct DNA methylation signature (high-CpG dinucleotide content) relative to other

genes in the genome [48]. Because *A. cephalotes* has more worker castes than other ant species [23] (Figure 1C), we predict that the DNA methylation signature of genes underlying wing polyphenism will also be distinct relative to other genes in its genome. To test this prediction, we analyzed the sequence composition of wing development genes in A. cephalotes, and found that they exhibit a higher CpG dinucleotide content than the rest of the genes in the genome (Text S10). Previous experiments have shown that genes with a high-CpG dinucleotide content can be differentially methylated in specific tissues or different developmental stages [49]. Therefore, DNA methylation may facilitate the caste-specific expression of genes that underlie wing polyphenism in A. cephalotes. This may be a general feature of genes that underlie polyphenism.

Desaturases

An important aspect of the eusocial lifestyle is communication between colony members, specifically in differentiating between individuals that belong to the same colony and those that do not. Nestmate recognition in many ants is mediated by cuticular hydrocarbons (CHCs) [50], and nearly 1,000 of these compounds have been described. In ants, CHC biosynthesis involves D9/D11 desaturases, which are known to produce alkene components of CHC profiles [51]. We analyzed the D9 desaturases in the genome of A. cephalotes and detected nine genes localized to a 200 kbp stretch on a single scaffold in addition to four other D9 desaturase genes on other scaffolds (Text S11). In contrast, the seven genes found in D. melanogaster are more widely distributed along one chromosome. The number of D9 desaturase genes in *A. cephalotes* is similar to the 9 and 16 found in *A. mellifera* and *N. vitripennis*, respectively. A phylogenetic analysis of these genes supports their division into five clades, with eight D9 desaturase genes

falling in a single clade suggesting an expansion of these genes possibly related to an increased demand for chemical signal variability during ant evolution (Text S11). Interestingly, the phylogeny also supports an expansion in this type of D9 desaturase genes within *N. vitripennis* but not in *A. mellifera*.

Immune Response

All insects have innate immune defenses to deal with potential pathogens [52] and A. cephalotes is no exception with a total of 84 annotated genes found to be involved in this response (Text S12). These include the intact immune signaling pathways Toll, Imd, Jak/Stat, and JNK. When compared to solitary insects like *D. melanogaster* and N. *vitripennis*, *A. cephalotes* has fewer immune response genes and better resembles what is known for the eusocial *A. mellifera* [53]. The presence of other defenses in A. cephalotes, such as antibiotics produced by metapleural glands [54–56], may account for the paucity of immune genes. Furthermore, social behavioral defenses may also participate in the immune response, as has been suggested for A. mellifera [53].

Chemosensory Genes

Chemosensation is an important aspect of *Atta cephalotes* biology, for both communication between colony members and between ants and their closely associated microbes including the fungus they cultivate for food and a protective bacterial symbiont. The chemosensory gene superfamily, which includes olfactory, gustatory & ionotropic receptors, has undergone numerous gene loses and expansions in the Hymenoptera. This divergence diminishes the utility of comparing this group with *Drosophila* orthologs (the baseline used to annotate many other *A.*

cephalotes genes). To facilitate this, we are taking advantage of the extensive chemosensory gene models compiled by the Robertson lab. I used the *Pogonomyrmex barbatus* olfactory receptors to annotate *A. cephalotes*. Annotation of some members of this diverse gene family was performed by combining manual annotation with computational predictions from MAKER. Annotations were centralized into a database and updated using Apollo (**Error! Reference source not found.**). This preliminary annotation revealed many orthologs, some with premature frameshifts or stop codons, likely to be pseudogenes. In other cases no obvious orthologs was found (genes failed to give one another as reciprocal best BLAST hits). As in other genomes, purely computational techniques to annotate chemosensory genes in the *Atta cephalotes* chemosensory genes remains inadequate to identify many of these genes without substantial manual annotation.

Orthology Analysis

A set of shared orthologs was determined among *A. cephalotes, A. mellifera, N. vitripennis*, and *D. melanogaster* (Figure 2). A total of 5,577 orthologs were found conserved across all four insect genomes, with an additional 1,363 orthologs conserved across the three hymenopteran genomes. A further, 599 orthologs were conserved between A. cephalotes and A. mellifera, perhaps indicating genes that are specific to a eusocial lifestyle. We also found 9,361 proteins that are unique to *A. cephalotes*, representing over half of its predicted proteome. These proteins likely include those specific to ants or to *A. cephalotes*.

We then analyzed the proteins that were found to be specific to *A. cephalotes* and determined those Gene Ontology (GO) [57] terms that are enriched in these proteins, relative to the rest of

the genome (Table S3). We found many GO terms that reflect the biology of A. cephalotes and ants in general. For example, we find proteins with GO terms that reflect the importance of communication. These include proteins associated with olfactory receptor activity, odorant binding function, sensory perception, neurological development, localization at the synapse, and functions involved in ligand-gated and other membrane channels.

Gene Comparison within Hymenopteran Genomes

To focus on Hymenoptera evolution, we compared the *A. cephalotes* genome to 4 other hymenopterans including the ants *C. floridanus* and *H. saltator*, the honey bee *A. mellifera*, and the solitary parasitic jewel wasp *N. vitripennis*. We used the eukaryotic clusters of orthologous groups (KOG) ontology [58] to annotate the predicted proteins from all of these genomes and performed an enrichment analysis by comparing the KOGs of the social insects *A. cephalotes, C. floridanus, H. saltator,* and *A. mellifera* against the KOGs of the non-social N. vitripennis as shown in Table S4.

A detailed analysis of KOGs within each over- and underrepresented category is highly suggestive of *A. cephalotes* biology (Table S5). One of the most over-represented KOGs in *A. cephalotes* includes the 69 copies of the RhoA GTPase effector diaphanous (KOG1924). In contrast, all of the other hymenopteran genomes have substantially less copies of this gene. RhoA GTPase diaphanous is known to be involved in actin cytoskeleton organization and is essential for all actin-mediated events [59]. The large number of these genes in *A. cephalotes* may relate to the extensive cytoskeletal changes that occur during caste differentiation. One of these genes (ACEP_00016791) was found to exhibit high single nucleotide polymorphism

(SNPs) (Text S13). Given that genes involved in caste development in other social insects like *A. mellifera* also have high SNPs [60,61], this may indicate that this gene is important for caste determination in *A. cephalotes*. *A. cephalotes* is also significantly over-represented in the dosage compensation complex subunit (KOG0921), the homeobox transcription factor SIP1 (KOG3623), the muscarine acetylcholine receptor (KOG4220), the cadhedrin EGF LAG seven-pass GTP-type receptor (KOG4289), and the calcium-activated potassium channel slowpoke (KOG1420), relative to *N. vitripennis*. Many of these genes have been implicated in D. melanogaster larval development, specifically during nervous system formation [62,63]. As a result, an over-representation of these genes in *A. cephalotes* relative to *N. vitripennis* may indicate their association with a eusocial lifestyle, and in particular, caste and subcaste differentiation.

Genes that were found to be under-represented in A. cephalotes relative to N. vitripennis include core histone genes, nucleosomebinding factor genes, serine protease trypsins, and cytochrome P450s (Table S5). These findings were confirmed by a domain-based comparison between A. cephalotes and all other sequenced insects (Text S14). One of the most under-represented KOGs is trypsin, a serine protease used in the degradation of proteins into their amino acid constituents. Trypsins in *N. vitripennis* are known to be part of the venom cocktail injected into its host, which helps necrotization and initiates the process of amino acid acquisition for developing larvae [35,64]. In contrast to the protein-rich diet of *N. vitripennis, A. cephalotes* feed on gongylidia produced by their fungus, which represents a switch to a carbohydrate-rich (60% of mixture) diet [65]. These differences in diet may explain the underrepresentation of trypsin in A. cephalotes, as trypsin is likely not the primary mechanism used to digest nutrients obtained from the fungal

cultivar. Our analysis also revealed a reduction of trypsin genes in the other social insects relative to *N. vitripennis*, and this may also reflect their diets. For example, honey dew is a major component of the diet of C. floridanus and contains primarily sugars [1], while the honey/pollen diet of A. mellifera is composed primarily of carbohydrates, lipids, carbohydrates, vitamins, and some proteins [66]. Because this under-representation of trypsin is consistent across social insects when compared to other sequenced insects (Table S5, Text S14), this reduction may reflect the specific dietary features of these insects, or could indicate a loss of these genes across eusocial insects.

In addition to trypsin, cytochrome P450s were also found to be under-represented in both *A. cephalotes* and *A. mellifera*, relative to N. *vitripennis*, with reductions in both CYP3- and CYP4-type P450s (Table S5). P450s in insects are important enzymes known to be involved in a wide range of metabolic activities, including xenobiotic degradation, and pheromone metabolism [67]. We identified a total of 52 and 62 P450s in *A. cephalotes* and *A. mellifera*, respectively, which is similar to the low numbers reported for another insect, the body louse *Pediculus humanus* [29]. These values represent some of the smallest amounts of P450s reported for any insect genome, and may represent the minimal number of P450s required by insects to survive. Comparison of the *A. cephalotes* P450s against those of *A. mellifera* and *P. humanus* reveals that while there are some shared P450s, many are specific to each insect (Text S15). In *A. mellifera*, the paucity of P450s is thought to be associated with the evolutionary underpinnings of its eusocial lifestyle [68], although an enrichment of P450s in the ants *C. floridanus* and *H. saltator* [27] would seem to contradict this prediction. It is therefore unclear why A. cephalotes has a small number of P450s relative to other ants, and future work will be necessary to provide insight into this

apparent discrepancy. A SNP analysis of the P450 genes in *A. cephalotes* did reveal that one of these, ACEP_00016463, has 20 SNPs/kbp (Text S13). Since P450s are known to undergo accelerated duplication and divergence [67], the high number of SNPs in this particular P450 may reflect positive selection for new functions.

Comparative Metabolic Reconstruction Analysis

Given the tight obligate association that A. cephalotes has with its fungal mutualist, one might predict that it acquires amino acids from its fungus in a manner similar to that of the pea aphid *Acyrthosiphon pisum*, which obtains amino acids from its bacterial symbionts [28]. To test this, we performed a metabolic reconstruction analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [69]. *A. cephalotes* contains a nearly identical set of amino acid biosynthesis genes as *A. mellifera*, *C. floridanus*, *H. saltator*, and *N. vitripennis*, all of which are incapable of synthesizing histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine *de novo*. The only exception is arginine, and only *A. cephalotes* was found to lack the genes necessary for its biosynthesis (Figure 3). Arginine, which is produced through the conversion of citrulline and aspartate [70,71], is predicted to be synthesized at levels too low to support growth in insects [72].

In *A. cephalotes* the 2 genes that catalyze the synthesis of arginine, argininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1), were not found (Figure 3). The loss of these two genes suggests a dependence on externally-acquired arginine, which we hypothesize, is provided by their fungus. In the carpenter ant *C. floridanus*, arginine is thought to be synthesized from citrulline provided by its endosymbiont *Blochmannia floridanus* [73], and this dependency

is predicted to play an essential role in maintaining the carpenter ant-bacteria mutualism. An extreme case has been reported for the pea aphid, which has lost its urea pathway and depends entirely on its endosymbiont, *Buchnera aphidicola*, for arginine [28]. The loss of arginine biosynthesis in *Atta* may similarly be important for maintaining the leaf-cutter ant-fungus mutualism. In line with this prediction, the fungus the ants cultivate contains all of the amino acids that *A. cephalotes* can not synthesize, including arginine [65].

Comparison of Hexamerins

In addition to arginine biosynthesis, *A. cephalotes* may have also lost the need to rely on hexamerins as a source of amino acids during development. In many insects, hexamerin proteins are synthesized by developing larvae and used as amino acid sources during development into the adult stage [74]. Four hexamerins are commonly found across insects, including hex 70a, hex 70b, hex 70c, and hex 110. Comparison among the hymenopteran genomes reveals the presence of all hexamerins in varying copy number across all genomes except for *A. cephalotes*, which is missing hex 70c (Figure 4) (Text S16). In A. mellifera, hexamerins are expressed at different times, with hex 70a and hex 110 expressed during the larval, pupal and adult stage of workers, and hex 70b and hex 70c only expressed during the larval stage [74]. The specific expression of hex 70b and hex 70c in larvae may reflect the increased need for these nutrients during early development. Given that *A. cephalotes* larvae feed primarily on gongylidia, it is possible that amino acids supplemented by the fungus over the millions of years of this mutualism has relaxed selection for maintaining larval-stage hexamerins, and thus hex 70c may have been lost. Future expression analyses of these genes at different life stages, in different castes, and under different nutritional conditions will likely confirm and elucidate their role.

Conclusion

Here we have presented the first genome sequence for a fungus-growing ant and show that its genomic features potentially reflectits obligate symbiotic lifestyle and developmental complexity. An initial analysis of its genome reveals many characteristics that are similar to both solitary and eusocial insect genomes. One hypothesis, based on the obligate mutualism of *Atta cephalotes* and its fungus, is that its genome exhibits reductions related to this relationship. We have provided some evidence that *A. cephalotes* has gene reductions related to nutrient acquisition, and these losses may be compensated by the provision of these nutrients from the fungus. For example, the extensive reduction in serine proteases may reflect the lack of proteins in its diet since the fungus primarily provides nutrients in the form of carbohydrates and free amino acids. Furthermore, the loss of the arginine biosynthesis pathway in *A. cephalotes* may indicate the obligate reliance that it has on the fungus, as arginine is part of the nutrients that it provides to the ant. This type of relationship appears to be conserved in other insect-microbe mutualisms, specifically in the pea aphid [28] and the carpenter ant [73]. Finally, *A. cephalotes* appears to have lost a hexamerin protein that is conserved across all other insect genome sequences reported to date. Loss of this protein, which is associated with amino acid sequestration during larval development, may be tolerated because larvae have a ready source of amino acids from the fungus. These genomic features may serve as essential factors that have stabilized the mutualism over its coevolutionary history. The sequencing and analysis of this genome will be a valuable addition to the growing number of insect genomes, and in particular will provide insight into both host-microbe symbiosis and eusociality in hymenopterans.

V. Materials and Methods

Sample Collection, DNA Extraction, and Sequencing

Three males from a single mature *Atta cephalotes* colony were collected in June 2009 in Gamboa, Panama (latitude 9u 79 00 N, longitude 79u 429 00 W) and designated males A, B, and C. Genomic DNA from these males was extracted using a modified version of a Genomic-tip extraction protocol for mosquitoes and other insects (QIAGEN, Valencia, CA). Sequencing was performed using the 454 FLX Titanium pyrosequencing platform [25] at the 454 Life Sciences Sequencing Center (Branford, CT) as follows. A whole-genome shotgun fragment library was constructed for male A and sequenced using a single run, generating 539,113,701 bp of sequence. For male B, a whole-genome shotgun fragment library was also constructed and sequenced using 11 runs, generating a total of 4,209,396,304 bp of sequence. An 8 kbp insert paired-end library was also generated for male B and sequenced using two runs, generating a total of 818,851,400 bp of sequence. A 20 kbp paired-end library was generated for male C, and sequenced using a single run, generating 349,435,001 bp. In total, 5,916,796,406 bp of sequence were generated for all three ants.

Genome Assembly

All generated sequences were assembled using the 454 GS *DE NOVO* assembler software (March 06 2010 R&D Release). The *Atta cephalotes* whole genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the project number 48117 and accession ADTU00000000. The version described in this paper is the first version, ADTU01000000. Transcript Sequencing and Assembly Workers from a healthy *Atta cephalotes* colony (JS090510-01) collected from Gamboa, Panama and maintained in the laboratory of Cameron Currie at the University of Wisconsin- Madison were used to generate transcript sequences. A pool of 169 workers across

different age and size classes was selected and total RNA was extracted using a modified version of a phenolchloroform protocol previously described [75]. This sample was normalized and a fragment library was generated before subsequent sequencing using a single run of a 454 FLX Titanium pyrosequencer [25] at the Genome Center at Washington University (St. Louis, MO), generating a total of 462,755,799 bp of sequence. Transcript sequences were assembled using the Celera assembler (wgs-assembler 6.0 beta) [76] with standard assembly parameters.

Genome Annotation

Annotations for the Atta cephalotes genome was generated using the automated genome annotation pipeline MAKER [32]. The MAKER annotation pipeline consists of 4 general steps. First, RepeatMasker (http://www.repeatmasker.org) and RepeatRunner [77] were used to identify and mask repetitive elements in the genome. Second, gene prediction programs including Augustus [78], Snap [79], and GeneMark [80] were employed to generate ab-initio (non-evidence informed) gene predictions. Next a set of expressed sequence tags (ESTs) and proteins from related organisms were aligned against the genome using BLASTN and BLASTX [81], and these alignments were further refined with respect to splice sites using the computer program Exonerate [82]. Finally, the EST and protein homology alignments and the *abinitio* gene predictions were integrated and filtered by MAKER to produce a set of evidence informed gene annotations. This gene set was then further refined to remove all putative repeat elements and to include gene models initially rejected by MAKER but found to contain known protein domains using the program InterProScan [83]. The resulting gene set (OGS 1.1) then became the substrate for further analysis and manual curation. Over 500 genes in OGS 1.1 were manually curated (Table S1), producing OGS 1.2, which is publicly available at the Hymenopteran Genome

Database (http://HymenopteraGenome.org/atta/genome_consortium).

The general manual curation process used for generating OGS 1.2 was based on a standardized protocol and conducted as follows. For each gene family, query sequences were obtained first from FlyBase [84] and supplemented with known gene models from the other sequenced hymenopteran genomes, *Apis mellifera* [26] and *Nasonia vitripennis* [35]. BLAST was used to align these gene models against putative sequences in the *A. cephalotes* genome predicted by MAKER. The sequence analysis program Apollo [85] was then used by all annotators to contribute their annotations to a centralized Chado [86] database. In general, putative gene models in A. cephalotes were confirmed by investigating the placement of introns and exons, the completeness of sequences, evaluating sequencing errors, and syntenic information. A final homology search was also performed with the putative A. cephalotes gene model by comparing it against the non-redundant protein database in NCBI to confirm its match against known insect models.

Repeat Analysis

We used PILER-DF [87], RepeatModeler (Smit A, Hubley R, Green P. RepeatModeler Open-1.0. 2008-2010 http://www.repeatmasker.org), RECON [88], and RepeatScout [89] to generate *de novo* transposable element (TE) predictions. We found 1,381 *de novo* repeat predictions including 264 from RepeatScout, 26 from PILER-DF, and 1091 from RECON. We simplified the complexity of our *de novo* TE predictions by removing elements that were over 80% similar over 80% of their length [90] and also screened out elements with more than 50% sequence identify to Uniprot [91] genes. This resulted in a final *A. cephalotes*-specific repeat library

containing 1,252 elements (1048 RECON, 195 RepeatScout, 9 PILER-DF), which were then classified using RepeatMasker (Smit AFA, Hubley R, Green P. RepeatMasker Open-3.0. 1996-2010 ,http://www.repeatmasker.org.) and custom scripts that identify TIR and LTR sequences. This curated library was converted to EMBL format, appended to a RepBase [92] library and used to mask the *A. cephalotes* genome assembly.

Orthology Analysis

An orthology analysis was performed between the proteins from *Atta cephalotes* (OGS1.2), *Apis mellifera* (preOGS2) [26], *Nasonia vitripennis* (OGSI 1.2) [35], and *Drosophila melanogaster* (Release 5.29) [93]. Using these protein sets, we reduced each dataset to contain only the single longest isoform using custom Perl scripts. An all-by-all BLAST was performed using the computer program OrthoMCL [94] and the best reciprocal orthologs, inparalogs, and co-orthologs were determined. We used the MCL v09-308 Markov clustering algorithm [95] to define final ortholog, inparalog, and co-ortholog groups between the datasets. For all OrthoMCL analyses, the suggested parameters were used.

We then annotated those proteins in *A. cephalotes* that did not have any orthologs to the 3 other insects and performed a gene ontology enrichment analysis. This was done by annotating all *A. cephalotes* proteins using Interproscan [83] to generate Gene Ontology (GO) [57] terms. This resulted in 6,971 (41%) proteins receiving at least one GO annotation. GO-TermFinder [96] was then used to determine those proteins that were enriched for specific GO terms in the A. cephalotes-specific proteins, relative to the entire *A. cephalotes* OGS1.2 dataset.

KOG Enrichment Analysis

We performed a eukaryotic orthologous groups (KOG) [58] enrichment analysis for the genomes of *Atta cephalotes, Camponotus floridanus* [27], *Harpegnathos saltator* [27], *Apis mellifera* [26], and *Nasonia vitrepennis* [35]. The KOG database was obtained from NCBI and RPSBLAST [97] (e-value: 1e-05) was used to comparethe predicted proteins from *A. cephalotes* (OGS1.2), *C. floridanus* (OGS3.3), *H. saltator* (OGS3.3), *A. mellifera* (preOGS2), and *Nasonia vitripennis* (OGS r.1). Each KOG hit was tabulated according to its gene category, and Fisher's exact test was then applied to determine which categories were over- or underrepresented. This was done for *A. cephalotes, C. floridanus, H. saltator*, and *A. mellifera* against *N. vitripennis*, respectively, as shown in Table S4. We then determine for each over- and underrepresented KOG category in *A. cephalotes* relative to *N. vitripennis*, the specific KOGs within each category that were significantly enriched or under-enriched. This was done by comparing the total number of *A. cephalotes* KOGs within each of these categories against those in N. vitripennis using Fisher's exact test, as shown in Table S5.

KEGG Reconstruction Analysis

The predicted peptides for *Atta cephalotes* were used to reconstruct putative metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes [69]. This was performed using the KEGG Automated Annotation Server (KAAS), which annotates proteins according to the KEGG database and reconstructs full pathways displaying them as maps. Similar maps were also constructed using KAAS for the predicted peptide sequences of *Camponotus floridanus* (OGS3.3) and *Harpegnathos saltator* (OGS3.3). These maps were compared against the maps currently available in KEGG for *Apis mellifera*, *Drosophila melanogaster*, and *Nasonia vitripennis*. For proteins in *A. cephalotes* that were not found in our KEGG reconstruction

analysis, relative to other insects (*e.g*. argininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1)), we investigated those reads that were not incorporated into the *A. cephalotes* assembly to confirm that these did not contain potential gene fragments corresponding to these genes.

List of Supporting Informaiton (available online)

Supporting Tables:
Table S1 Total number of genes annotated in the *A. cephalotes* genome according to gene family
Table S2 Repetitive elements identified in the *A. cephalotes* genome
Table S3 Gene Ontology enrichment of proteins specific to *A. cephalotes* relative to *A. mellifera*, *D. melanogaster*, and *N. vitripennis*
Table S4 Enrichment comparison of proteins in categories of KOGs for Atta cephalotes, *Camponotus floridanus*, *Harpegnathos saltator*, and *Apis mellifera* relative to *Nasonia vitripennis*
Table S5 Over- and under-represented KOGs in *Atta cephalotes* (Acep), *Camponotus floridanus* (Cflo), *Harpegnathos saltator* (Hsal), and *Apis mellifera* (Amel) compared to *Nasonia vitripennis* (Nvit) according to category

Supporting Texts:
Text S1 Cytoplasmic Ribosomal Proteins
Text S2 Evaluating Sequence Quality and Coverage using Cytoplasmic Ribosomal Proteins
Text S3 Oxidative Phosphorylation Proteins
Text S4 The *Atta cephalotes* Mitochondrial Genome
Text S5 Global Composition Analysis
Text S6 DNA Methylation Tool Kit
Text S7 RNAi Genes
Text S8 Insulin Signalling Pathway Genes
Text S9 Yellow/Major Royal Jelly Protein Family
Text S10 Wing Polyphenism Gene Networks
Text S11 The D 9 Desaturase Genes
Text S12 Immune Genes
Text S13 SNP Analysis
Text S14 Protein Domain Contraction and Expansion
Text S15 Cytochrome p450s
Text S16 Hexamerins
Text S17 Alien Gene Fragments
Text S18 Chitinase Genes
Text S19 Hox Genes
Text Micro RNAs

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VII. References

1. Hölldobler B, Wilson EO (1990) The ants. CambridgeMass: Harvard University Press. 732 p.

2. Belt T (1874) The naturalist in Nicaragua. London: E. Bumpus. 306 p.

3. Weber NA (1966) Fungus-growing ants. Science 153: 587–604.

4. Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. Proc Natl Acad Sci U S A 105: 5435.

5. Fowler G, Forri L (1986) Economics of grass-cutting ants. In: Lofgren C, Vander Meer R, eds. Fire ants and leaf cutting ants: biology and management. BoulderCO: Westview Press. pp 123–145.

6. Hölldobler B, Wilson EO (2008) The superorganism: the beauty, elegance, and strangeness of insect societies. New York: W.W. Norton. 544 p.

7. Wirth R, Herz H, Ryel RJ, Beyschlag W, Hölldobler B (2003) Herbivory of leafcutting ants. A case study on Atta colombica in the tropical rain forest of Panama. Berlin, Heidelberg: Springer. 230 p.

8. Currie CR (2001) A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. Annu Rev Microbiol 55: 357–380.

9. Mueller UG, Schultz TR, Cameron RC, Adams RMM, Malloch D (2001) The origin of the attine ant-fungus mutualism. Q Rev Biol 76: 169–197.

10. Pinto-Tomas AA, Anderson MA, Suen G, Stevenson DM, Chu FST, et al. (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science 326: 1120–1123.

11. Suen G, Scott JJ, Aylward FO, Adams SM, Tringe SG, et al. (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. PLoS Genet 6: e1001129. doi:10.1371/journal.pgen.1001129.

12. Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, et al. (2003) Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299: 386–388.

13. Currie CR (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128: 99–106.

14. Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398: 701–704.

15. Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101: 91–102.

16. Oh D-C, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nat Chem Biol 5: 391–393.

17. Little AE, Currie CR (2007) Symbiotic complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. Biol Lett 3: 501–504.

18. Little AE, Currie CR (2008) Black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. Ecology 89: 1216–1222.

19. Frost CL, Fernandez-Marin H, Smith JE, Hughes WOH (2010) Multiple gains and losses of Wolbachia symbionts across a tribe of fungus-growing ants. Mol Ecol 19: 4077–4085.

20. Pagnocca FC, Legaspe MFC, Rodrigues A, Ruivo CCC, Nagamoto NS, et al. (2010) Yeasts isolated from a fungus-growing ant nest, including the description of *Trichosporon chiarellii* sp. nov., an anamorphic basidiomycetous yeast. Int J Syst Evol Microbiol 60: 1454–1459.

21. Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis. Proc Natl Acad Sci U S A 106: 4742–4746.

22. Bacci M, Jr., Ribeiro SB, Casarotto MEF, Pagnocca FC (1995) Biopolymerdegrading bacteria from nests of the leaf-cutting ant *Atta sexdens rubropilosa*. Braz J Med Biol Res 28: 79–82.

23. Wilson EO (1983) Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*): IV. colony ontogeny of *A. cephalotes*. Behav Ecol Sociobiol 14: 55–60.

24. Wilson EO (1983) Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta)*: III. ergonomic resiliency in foraging by *A. cephalotes*. Behav Ecol Sociobiol 14: 47–54.

25. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380.

26. Honey Bee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.

27. Bonasio R, Zhang G, Ye C, Mutti NS, Fang X, et al. (2010) Genomic comparison of the Ants *Camponotus floridanus* and *Harpegnathos saltator*. Science 329: 1068–1071.

28. The International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol 8: e1000313. doi:10.1371/journal.pbio.1000313.

29. Kirkness EF, Haas BJ, Sun W, Braig HR, Perotti MA, et al. (2010) Genome sequences of the human body louse and its primary endosymbiont providen sights into the permanent parasitic lifestyle. Proc Natl Acad Sci U S A 107:12168–12173.

30. Tsutsui N, Suarez A, Spagna J, Johnston JS (2008) The evolution of genome size in ants. BMC Evol Biol 8: 64.

31. Parra G, Bradnam K, Korf I (2007) CEGMA: a pipeline to accurately annotatecore genes in eukaryotic genomes. Bioinformatics 23: 1061–1067.

32. Cantarel BL, Korf I, Robb SMC, Parra G, Ross E, et al. (2008) MAKER: An easy-to-use annotation pipeline designed for emerging model organism genomes. Genome Res 18: 188–196.

33. Wei S, Shi M, He J, Sharkey M, Chen X (2009) The complete mitochondrial genome of *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) indicates extensive independent evolutionary events. Genome 52: 308–319.

34. Crozier RH, Crozier YC (1993) The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics 133: 97–117.

35. Werren JH, Richards S, Desjardins CA, Niehuis O, Gadau J, et al. (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. Science 327: 343–348.

36. Karlin S, Mrazek J (1997) Compositional differences within and between eukaryotic genomes. Proc Natl Acad Sci U S A 94: 10227–10232.

37. Wang Y, Jorda M, Jones PL, Maleszka R, Ling X, et al. (2006) Functional CpG methylation system in a social insect. Science 314: 645–647.

38. Feng S, Cokus SJ, Zhang X, Chen P-Y, Bostick M, et al. (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci U S A 107: 8689–8694.

39. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328: 916–919.

40. Field LM, Lyko F, Mandrioli M, Prantera G (2004) DNA methylation in insects. Insect Mol Biol 13: 109–115.

41. Kucharski R, Maleszka J, Foret S, Maleszka R (2008) Nutritional control of reproductive status in honeybees via DNA methylation. Science 319: 1827–1830.

42. Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431: 343–349.

43. Forstemann K, Tomari Y, Du T, Vagin VV, Denli AM, et al. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 3: e236. doi:10.1371/journal.pbio.0030236.

44. Wu Q, Brown MR (2006) Signaling and function of insulin-like peptides in insects. Annu Rev Entomol 51: 1–24.

45. de Azevedo SV, Hartfelder K (2008) The insulin signaling pathway in honey bee (*Apis mellifera*) caste development - differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. J Insect Physiol 54: 1064–1071.

46. Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the yellow/major royal jelly protein family and the emergence of social behavior in honey bees. Genome Res 16: 1385–1394.

47. Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. Science 297: 249–252.

48. Elango N, Hunt BG, Goodisman MAD, Yi SV (2009) DNA methylation is widespread and associated with differential gene expression in castes of the honeybee, *Apis mellifera*. Proc Natl Acad Sci U S A 106: 11206–11211.

49. Illingworth R, Kerr A, DeSousa D, Jurgensen H, Ellis P, et al. (2008) A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol 6: e22. doi:10.1371/journal.pbio.0060022.

50. Wagner D, Tissot M, Cuevas W, Gordon DM (2000) Harvester ants utilize cuticular hydrocarbons in nestmate recognition. J Chem Ecol 26: 2245–2257.

51. Martin S, Drijfhout F (2009) A review of ant cuticular hydrocarbons. J Chem Ecol 35: 1151–1161.

52. Vilmos P, Kurucz E ´ (1998) Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol Lett 62: 59–66.

53. Evans JD, Aronstein K, Chen YP, Hetru C, Imler JL, et al. (2006) Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Mol Biol 15: 645–656.

54. Do Nascimento RR, Schoeters E, Morgan ED, Billen J, Stradling DJ (1996) Chemistry of metapleural gland secretions of three attine ants, *Atta sexdens rubropilosa, Atta cephalotes*, and *Acromyrmex octospinosus* (Hymenoptera: Formicidae). J Chem Ecol 22: 987–1000.

55. Poulsen M, Hughes WOH, Boomsma JJ (2006) Differential resistance and the importance of antibiotic production in Acromyrmex echinatior leaf-cutting ant castes towards the entomopathogenic fungus Aspergillus nomius. Insect Soc 53: 349–355.

56. Fernandez-Marin H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the metapleural glands by ants in controlling fungal infection. Proc R Soc London Ser B Biol Sci 273: 1689–1695.

57. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene Ontology: tool for the unification of biology. Nat Genet 25: 25.

58. Tatusov R, Fedorova N, Jackson J, Jacobs A, Kiryutin B, et al. (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4: 41.

59. Wasserman S (1998) FH proteins as cytoskeletal organizers. Trends in Cell Biol 8: 111–115.

60. Smith CR, Toth AL, Suarez AV, Robinson GE (2008) Genetic and genomic analyses of the division of labour in insect societies. Nat Rev Genet 9: 735–748.

61. Beye M, Hasselmann M, Fondrk MK, Page Jr. RE, Omholt SW (2003) The gene csd is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell 114: 419–429.

62. Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, et al. (1993) *Drosophila* nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 44: 716–724.

63. Becker MN, Brenner R, Atkinson NS (1995) Tissue-specific expression of a *Drosophila* calcium-activated potassium channel. J Neurosci 15: 6250–6259.

64. De Graaf DC, Aerts M, Brunain M, Desjardins CA, Jacobs FJ, et al. (2010) Insights into the venom composition of the ectoparasitoid wasp *Nasonia vitripennis* from bioinformatic and proteomic studies. Insect Mol Biol 19: 11–26.

65. Martin MM, Carman RM, Macconnell JG (1969) Nutrients derived from the fungus cultured by the fungus-growing ant Atta colombica tonsipes. Ann Ent Soc Am 62: 11–13.

66. Haydak MH (1970) Honey bee nutrition. Ann Rev Entomol 15: 143–156.

67. Feyereisen R (1999) Insect P450 enzymes. Annu Rev Entomol 44: 507–533.

68. Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, et al. (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol Biol 15: 615–636.

69. Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M (2009) KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res 38: D355–D360.

70. Hinton T, Noyes DT, Ellis J (1951) Amino acids and growth factors in a chemically defined medium for Drosophila. Physiol Zool 24: 335–353.

71. Reddy SRR, Campbell JW (1969) Arginine metabolism in insects: properties of insect fat body arginase. Comp Biochem Physiol 28: 515–534.

72. Raghupathi Rami Reddy S, Campbell J (1977) Enzymic basis for the nutritional requirement of arginine in insects. Cell Mol Life Sci 33: 160–161.

73. Gil R, Silva FJ, Zientz E, Delmotte F, Gonzalez-Candelas F, et al. (2003) The genome sequence of *Blochmannia floridanus*: Comparative analysis of reduced genomes. Proc Natl Acad Sci U S A 100: 9388–9393.

74. Martins J, Nunes F, Cristino A, Simoes Z, Bitondi M (2010) The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. BMC Mol Biol 11: 23.

75. Chen J, Weimer PJ (2001) Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria. Microbiology 147: 21–30.

76. Miller JR, Delcher AL, Koren S, Venter E, Walenz BP, et al. (2008) Aggressive assembly of pyrosequencing reads with mates. Bioinformatics 24: 2818–2824.

77. Smith CD, Edgar RC, Yandell MD, Smith DR, Celniker SE, et al. (2007) Improved repeat identification and masking in Dipterans. Gene 389: 1–9.

78. Stanke M, Tzvetkova A, Morgenstern B (2006) AUGUSTUS at EGASP: using EST, protein and genomic alignments for improved gene prediction in the human genome. Genome Biol 7: S11.

79. Korf I (2004) Gene finding in novel genomes. BMC Bioinformatics 5: 59.

80. Lukashin AV, Borodovsky M (1998) GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res 26: 1107–1115.

81. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

82. Slater G, Birney E (2005) Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics 6: 31.

83. Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, et al. (2009) InterPro: the integrative protein signature database. Nucleic Acids Res 37: D211–D215.

84. Tweedie S, Ashburner M, Falls K, Leyland P, McQuilton P, et al. (2009) FlyBase: enhancing Drosophila gene ontology annotations. Nucleic Acids Res 37: D555–D559.

85. Lewis SE, Searle SMJ, Harris N, Gibson M, Iyer V, et al. (2002) Apollo: a sequence annotation editor. Genome Biol 3: RESEARCH0082.

86. Mungall CJ, Emmert DB, The FlyBase Consortium (2007) A Chado case study: an ontology-based modular schema for representing genome-associated biological information. Bioinformatics 23: i337–i346.

87. Edgar RC, Myers EW (2005) PILER: identification and classification of genomic repeats. Bioinformatics 21: i152–i158.

88. Bao Z, Eddy SR (2002) Automated *de novo* identification of repeat sequence families in sequenced genomes. Genome Res 12: 1269–1276.

89. Price AL, Jones NC, Pevzner PA (2005) *De novo* identification of repeat families in large genomes. Bioinformatics 21: i351–i358.

90. Wicker T, Sabot F, Hua-Van A, Bennetzen JL, Capy P, et al. (2007) A unified classification system for eukaryotic transposable elements. Nat Rev Genet 8: 973.

91. The UniProt Consortium (2010) The universal protein resource (UniProt) in 2010. Nucleic Acids Res 38: D142–D148.

92. Jurka J, Kapitonov V, Pavlicek A, Klonowski P, Kohany O, et al. (2005) Repbase update, a database of eukaryotic repetitive elements. Cytogenet Genome Res 110: 462–467.

93. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, et al. (2000) The genome sequence of *Drosophila melanogaster*. Science 287: 2185–2195.

94. Li L, Stoeckert CJ, Roos DS (2003) OrthoMCL: identification of orthologs groups for eukaryotic genomes. Genome Res 13: 2178–2189.

95. van Dongen S (2000) Graph clustering by flow simulation: Phd Thesis, Universiteit Utrecht.

96. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, et al. (2004) GO::TermFinder - open source software for accessing gene ontology information and finding significantly enriched gene ontology terms associated with a list of genes. Bioinformatics 20: 3710–3715.

97. Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, et al. (2007) CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res 35: D237–D240

VIII. TABLES

Table 1. General assembly statistics for the genome of the leaf-cutter ant *Atta cephalotes*

Statistic	*Atta cephalotes*
Number of contigs	42,754
Average length of contigs	6,788 bp
Total length of contigs	290,223,730 bp
Contig size N50	14,240 bp
Number of scaffolds	2,835
Average length of scaffolds	112,054 bp
Total length of all scaffolds	317,672,992 bp
Scaffold size N50	5,154,504 bp
Total coverage	18-20X
Predicted genes (OGS1.2)	18,093

Table 2. Subset, *Atta cephalotes* olfactory gene annotations. Legend: Legend:7tm_6: 7 transmembrane domain protein type 6; Or: Olfactory receptor; PSE: Likely pseudogene;

A. cephalotes gene ID	*P. barbatus* ortholog	Scafford Number	Amino acid size	Conserved domain	Function
AcOr1	PbOr1	3	524	7tm_6	Or
AcOr2	PbOr2	5	420	7tm_6	Or
AcOr3	PbOr3	5	469	7tm_6	Or
-	PbOr4	-	-	7tm_6	Or
AcOr5	PbOr5	5	501	7tm_6	Or
AcOr6	PbOr6	5	410	7tm_6	Or
AcOr7PSE	PbOr7PSE	5	111	7tm_6	Or
AcOr8	PbOr8	5	360	7tm_6	Or
AcOr9	PbOr9	5	400	7tm_6	Or
AcOr10PSE	PbOr10	5	312	7tm_6	Or

IX. FIGURES

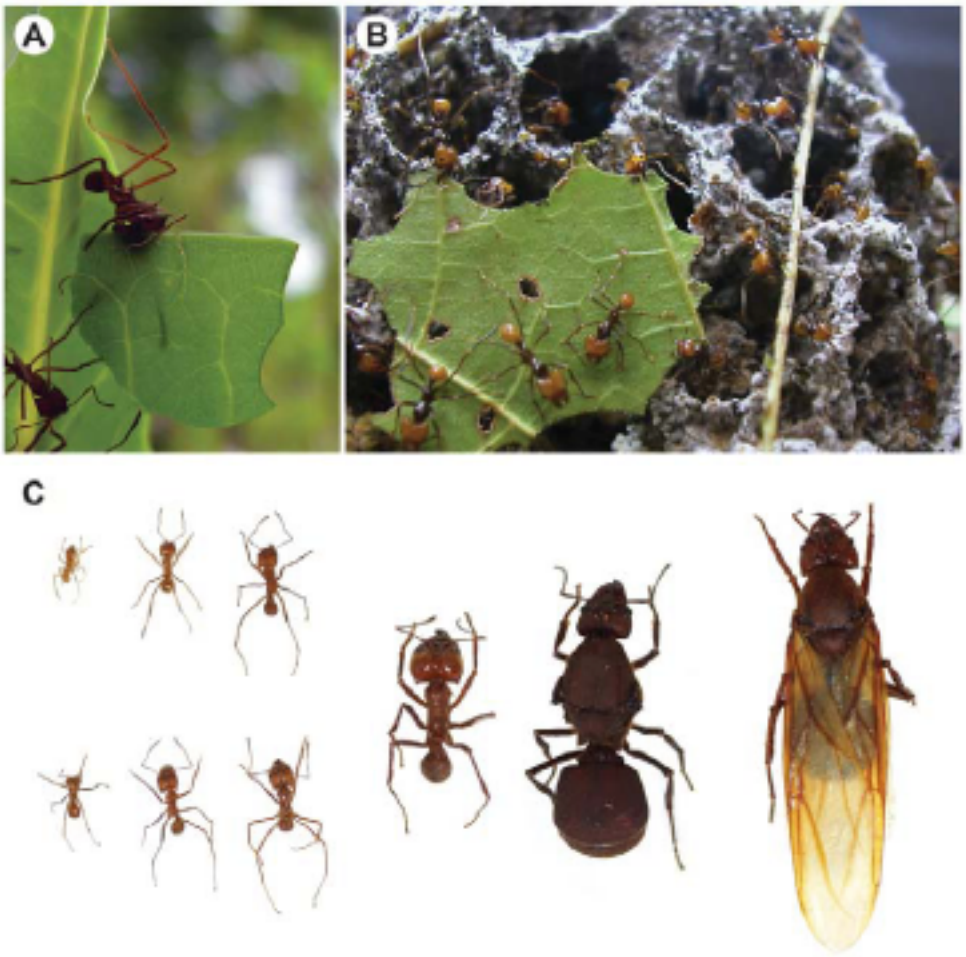

Figure 1. The leaf-cutter ant, *Atta cephalotes*. Leaf-cutter ants harvest fresh leaf material which they cut from Neotropical rainforests(a) and use them to grow a fungus that serves as the colony's primary food source (b). These ants display a morphologically diverse caste system that reflects a complex division of labor (c) correlated to specific tasks within the colony. These include small workers that undertake garden management and brood care, medium workers that forage leaves, large workers that can serve as soldiers, and winged sexuals that lose their wings after mating. [Photo Credits: foraging workers, Jarrod J. Scott/University of Wisconsin-Madison; fungus garden, Austin D. Lynch/ University of Wisconsin-Madison; caste morphology, used under the GNU Free Documentation License version 1.3].

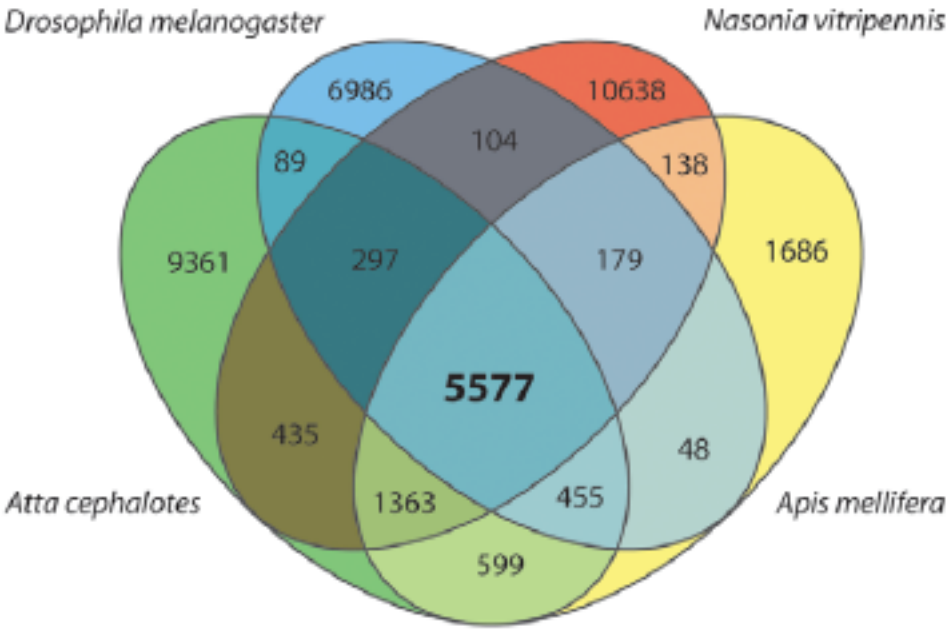

Figure 2. Orthology Analysis of the *Atta cephalotes* predicted peptide sequences (green) against the proteomes of the fly *Drosophila melanogaster* (blue), the wasp *Nasonia vitripennis* (red), and the honey bee *Apis mellifera* (yellow).

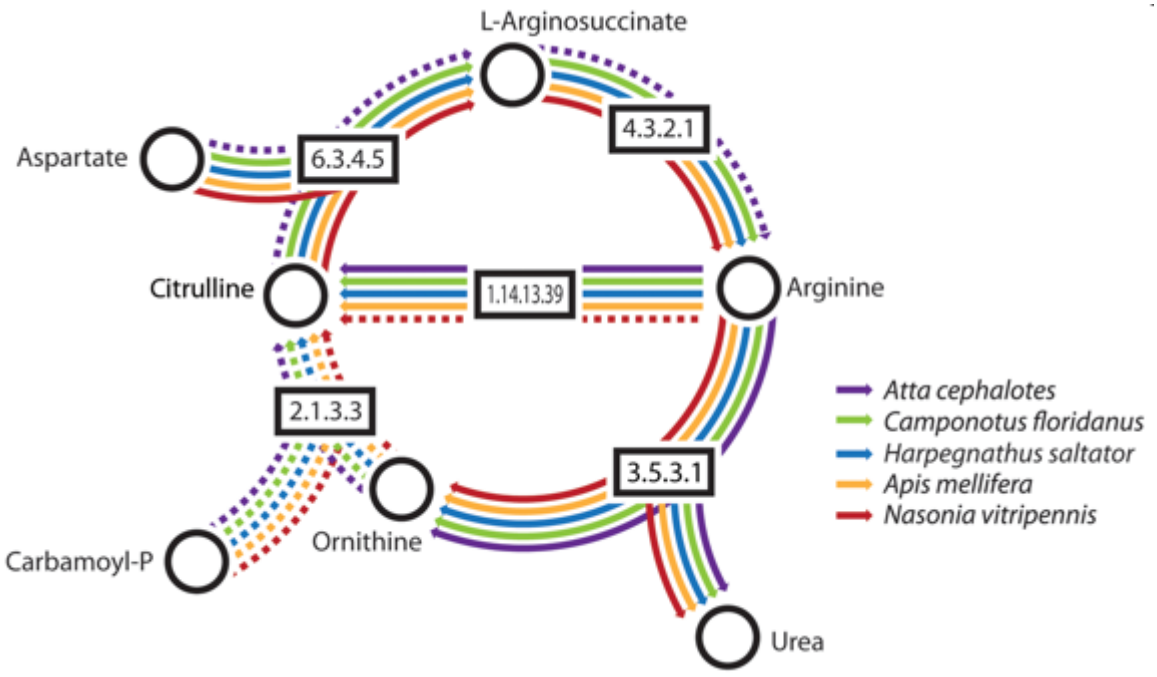

Figure 3. Predicted arginine biosynthesis pathway map in *Atta cephalotes*, *Camponotus floridanus*, *Harpegnathus saltator*, *Apis mellifera*, and *Nasonia vitripennis*. This pathway in A. cephalotes was found to be missing the two enzymes agininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1), which catalyzes the conversion of aspartate and citrulline into arginine. Other enzymes in this pathway include ornithine cabamoyltransferase (EC 2.1.3.3), arginase (EC 3.5.3.1) and nitric oxide synthase (EC 1.14.13.39). Dotted arrows indicate genes encoding proteins which were not found.

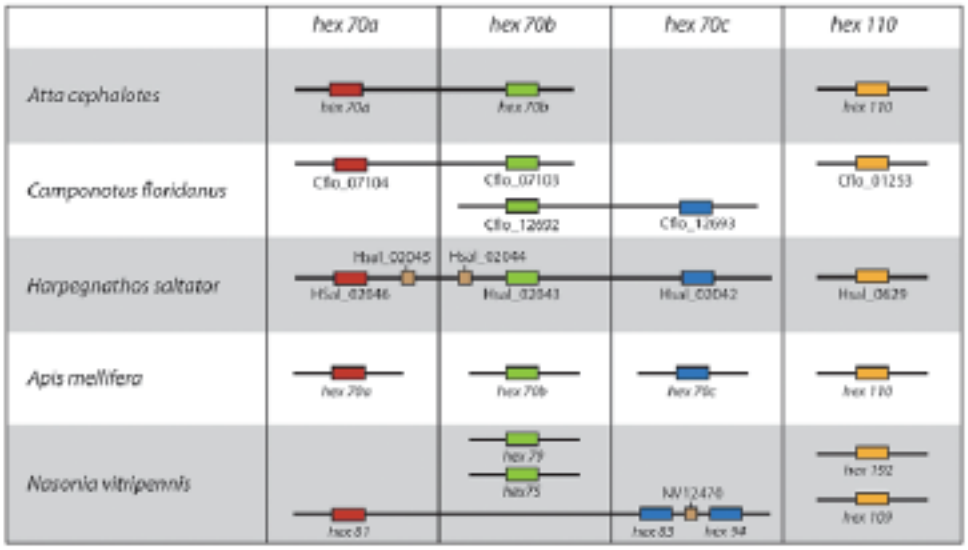

Figure 4. Distribution of hexamerin genes in the genomes of *Atta cephalotes*, *Camponotus floridanus*, *Harpegnathos saltator*, *Apis mellifera*, and *Nasonia vitripennis*. Four hexamerins with varying copy number are found within these genomes except for A. cephalotes which is missing hex 70c. Many of these genes are found to be syntenic along chromosomes/scaffolds, as shown (not drawn to scale).