

**THE ECOLOGY AND EVOLUTION OF A QUADRIpartite SYMBIOSIS:
EXAMINING THE INTERACTIONS AMONG ATTINE ANTS,
FUNGI, AND ACTINOMYCETES**

by

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ABSTRACT

The ecology and evolution of a quadripartite symbiosis: Examining the interactions among attine ants, fungi, and actinomycetes, Ph.D., 2000, Cameron Robert Currie, Department of Botany, University of Toronto

The ancient and highly evolved mutualism between fungus-growing ants (Formicidae: Attini) and their fungi (Agaricales: mostly Lepiotaceae) is a textbook example of symbiosis. The ants carefully tend the fungus, which serves as their main food source, and traditionally are believed to maintain the fungus free of microbial parasites. In this thesis, I conduct the first extensive examination of parasites attacking the fungus gardens of attine ants. I establish that the gardens are host to specialized and virulent parasitic fungi in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales), which can completely overwhelm gardens. Persistent infections of this fungus also result in decreases in the growth rate of infected colonies. In addition, I establish a completely new and fundamental mechanism employed by the ants to maintain the health of their fungal cultivars: a mutualistic association with actinomycetes (a group of filamentous bacteria that are well-known for their ability to produce antibiotics). The actinomycete is present in all species of fungus-growing ants examined, and is carried upon regions of the ants' cuticle that appear to be both specialized and genus-specific. I also found the bacterium to be present on queens during their mating flight, indicating that it is primarily vertically transmitted between colonies. Bioassays failed to detect the production of any general antifungal metabolites by the ant-associated bacterium, but revealed that potent metabolites are produced that target *Escovopsis*. Using a two-by-two factorial design experiment, crossing the presence/absence of actinomycete with the presence/absence of *Escovopsis*, I established that sub-colonies with the bacterium present were significantly more resistant to infections of

Escovopsis than those with the bacterium removed. It now appears that the attine symbiosis is a co-evolutionary 'arms race' between the garden parasite *Escovopsis*, on the one hand, and the tripartite association amongst the actinomycete, the ants, and the fungal mutualist on the other. The importance of both *Escovopsis* and the actinomycete in this mutualism suggests that microbes may mediate the interactions occurring in other mutualisms and suggests that studying the highly evolved chemical interactions occurring within this symbiosis may provide valuable theoretical and practical insights for the discovery and use of antibiotics.

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Table of Contents

Title Page.....	i
Abstract.....	ii
Acknowledgements.....	iv
Table of Contents	vii
List of Tables	x
List of Figures.....	xi
Chapter 1: General Introduction.....	1
1.1 Introduction.....	1
1.2 The Ecology and Evolution of Symbiosis.....	4
1.2.1 Evolutionary stability of mutualism.....	4
1.2.2 The symbiotic continuum.....	5
1.2.3 Virulence	6
1.2.4 Co-evolutionary 'arms races' and the Red Queen hypothesis.....	7
1.2.5 Parasites of mutualism	10
1.3 Mutualism Between Fungus-Growing Ants and Their Fungi.....	11
1.3.1 Introduction	11
1.3.2 Evolution of fungus-growing in attine ants.....	16
1.3.3 Ecology of fungus-growing in attine ants	22
1.3.4 Alien microbes in ant fungal gardens.....	27
1.4 Thesis Objectives	28
Chapter 2: The Agricultural Pathology of Ant Fungus Gardens	34
2.1 Abstract.....	34
2.2 Introduction.....	35
2.3 Materials and Methods.....	37
2.3.1 Sampling of attine gardens.....	37
2.3.2 Pathogenicity and virulence of garden contaminants.....	39
2.3.3 Transmission of <i>Escovopsis</i>	40
2.3.4 Intergeneric comparison	41
2.4 Results.....	41
2.4.1 Sampling of attine gardens.....	41
2.4.2 Pathogenicity and virulence of garden contaminants.....	42
2.4.3 Transmission of <i>Escovopsis</i>	43
2.4.4 Intergeneric comparison.....	43
2.5 Discussion	44

Chapter 3: Prevalence and Impact of a Virulent Parasite on a Tripartite Mutualism	54
3.1 Abstract	54
3.2 Introduction	55
3.3 Materials and Methods.....	58
3.3.1 Prevalence of <i>Escovopsis</i>	58
3.3.2 Age of colony and infections of <i>Escovopsis</i>	60
3.3.3 Impact of <i>Escovopsis</i> on colony growth rate	60
3.3.4 Within garden location of <i>Escovopsis</i>	62
3.4 Results.....	63
3.4.1 Prevalence of <i>Escovopsis</i>	63
3.4.2 Age of colony and presence of <i>Escovopsis</i>	63
3.4.3 Impact of <i>Escovopsis</i> on colony growth rate	63
3.4.4 Within garden location of <i>Escovopsis</i>	64
3.5 Discussion	64
Chapter 4: Fungus-Growing Ants Use Antibiotic Producing Bacteria to Control Garden Parasites	77
4.1 Abstract/Introduction	77
4.2 Results and Discussion.....	78
4.3 Methods.....	83
4.3.1 Identification of bacterium	83
4.3.2 Attines examined.....	83
4.3.3 Antibiotic bioassay challenges	84
4.3.4 Growth promotion bioassays.....	85
Chapter 5: Experimental Evidence of a Tripartite Mutualism: Bacteria Help Protect Leaf-Cutting Ant Fungal Gardens From Specialized Parasites	93
5.1 Abstract	93
5.2 Introduction	94
5.3 Methods.....	98
5.3.1 Study organisms	98
5.3.2 Experimental tests of actinomycete suppression of <i>Escovopsis</i>	99
5.3.3 Abundance of actinomycete on gardening versus foraging workers	103
5.3.4 Pathogen-induced actinomycete growth promotion.....	104
5.4 Results.....	105
5.4.1 Experimental tests of actinomycete suppression of <i>Escovopsis</i>	105
5.4.2 Abundance of actinomycete on gardening versus foraging workers	105
5.4.3 Pathogen-induced actinomycete growth promotion.....	106
5.5 Discussion	106

Chapter 6: General Discussion.....	123
6.1 Monoculture	124
6.2 <i>Escovopsis</i>	126
6.3 Biological Control	129
6.4 Red Queen and the Co-Evolutionary 'Arms Race'	130
6.5 Parasites of Mutualisms	131
6.6 Relevance for Human Survival	131
6.7 Conclusions.....	132
 Chapter 7: General Summary.....	 134
 Chapter 8: Literature Cited	 138

List of Tables

Table 1.1 Genera of fungus-growing ants	14 & 15
Table 2.1 Proportion of attine ant colonies parasitized by <i>Escovopsis</i> from sampling in Panama, 1996 to 1998.....	48
Table 2.2 Isolation of <i>Escovopsis</i> spp. from different life history stages of <i>A. colombica</i>	49
Table 3.1 Frequency of <i>Escovopsis</i> in colonies of leaf-cutting ant species from different locations in the canal region of Panama.....	69
Table 4.1 Location of <i>Streptomyces</i> on different genera of attine ants	86
Table 5.1 Abundance of actinomycete on workers and gynes in <i>A. octospinosis</i>	114

List of Figures

Figure 1.1 Evolutionary history of attine ants.....	33
Figure 2.1 Proportion of garden pieces containing non-mutualistic filamentous fungi.....	51
Figure 2.2 Fungal gardens of <i>Trachymyrmex sp.</i> : Healthy garden and garden overgrown by the parasite <i>Escovopsis</i>	53
Figure 3.1 Prevalence of <i>Escovopsis</i> in <i>Atta</i> spp. colonies by age of colony	71
Figure 3.2 Impact of <i>Escovopsis</i> on the growth rate of <i>A. colombica</i> colonies	73 & 74
Figure 3.3 Prevalence of <i>Escovopsis</i> in different locations of the infected gardens of <i>A. colombica</i>	76
Figure 4.1 Photograph illustrating the presence of the third mutualist, <i>Streptomyces</i> , on the cuticle of <i>A. octospinosus</i>	88
Figure 4.2 Scanning electron micrographs of fungus-growing ants showing the location of <i>Streptomyces</i>	90
Figure 4.3 Bioassay challenge between actinomycete and <i>Escovopsis</i>	92
Figure 5.1 The role of actinomycete in suppressing the growth of <i>Escovopsis</i> in <i>A. octospinosus</i> fungal gardens	116
Figure 5.2 Impact of actinomycete on garden growth.....	118
Figure 5.3 Abundance of actinomycete on workers tending the fungal garden versus foragers	120
Figure 5.4 Impact of infection by <i>Escovopsis</i> on abundance of actinomycete on workers	122

THE ECOLOGY AND EVOLUTION OF A QUADRIPARTITE
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1.0 GENERAL INTRODUCTION

1.1 INTRODUCTION

Symbiotic associations, including both parasitism and mutualism (see below), shape the evolution of every living organism and all levels of biological organization (Boucher *et al.* 1982, Boucher 1985, Price *et al.* 1986, Ahmadjian and Paracer 1986, Maynard Smith and Szathmáry 1995). Organisms do not occur in isolation; they live in close association with a phylogenetically diverse assemblage of other species, thereby establishing symbiosis as a ubiquitous and ecologically dominant component of the natural world. Mutualisms, for instance, once thought to be rare and therefore of limited importance (see Boucher *et al.* 1982, Boucher 1985), are now recognized as having shaped the evolution of higher life on earth. The importance of mutualism is illustrated by the fact that 90% of land plants depend on mycorrhizal fungi for nutrients, and that mammalian and insect herbivores depend on gut microbes to break down cellulose. Even eukaryotic cells evolved through symbiosis, as mitochondria and chloroplasts are believed to be endosymbiotic bacteria (Margulis and Fester 1991, Maynard Smith and Szathmáry 1995). Parasitic symbioses are likewise of vast biological importance. They are thought to drive the population dynamics of many organisms and mediate other interspecific interactions, including competition and predation (Feener 1981, Freeland 1983, Price *et al.* 1986, Yan *et al.* 1998). Even the dominance of

to be maintained through the presence of parasites (Jaenike 1978, Hamilton 1980, Hamilton *et al.* 1990).

As recently pointed out by Bull and Wichman (1998) and Thompson (1999) the study of the ecology and evolution of symbiosis is not only fundamental for understanding the world and our place within it, but it is increasingly of critical importance for human welfare. For example, an understanding of the evolution of parasites, particularly in regard to their virulence and transmission, has important applications in human medicine and agriculture. In fact, attempts to apply theoretical models of virulence evolution in parasites to human medical practices has led to the development of a whole new field over the last decade: Darwinian Medicine (Profet 1991, Williams and Nesse 1991, Profet 1993, Ewald 1994). Although there has been promising theoretical progress in this field (e.g., Ewald 1994), little of the generated knowledge and ideas has penetrated mainstream medicine. Nevertheless, with the emerging crisis of human diseases developing resistance to antibiotics and the higher prevalence of immunosuppressed individuals, understanding the evolution of virulence is obviously of vast importance. In addition, the future success of human agriculture will become increasingly dependent on our ability to manage pests and pathogens; an understanding of how these evolve as symbionts will help us develop better methods for their management.

The term 'symbiosis' was first coined in 1877 by Albert Bernhard Frank (Sapp 1994) for "two different species that live on or in one another". He specifically intended it to be a neutral term, so that the relationship occurring between two organisms is not prejudged by the terminology. Thus the term 'symbiosis' is useful for referring to all close associations of phylogenetically

distantly related organisms, from parasitism to mutualism. In 1879, Anton de Bary, who is often falsely credited with coining the term symbiosis (e.g., Lewis 1985, Ahmadjian and Paracer 1986), defined it as "the living together of unlike named organisms". More recently, some biologists have restricted the term symbiosis to only mutualistic associations. However, based on the original definition by both Frank and de Bary, it is clear that symbiosis should include parasitism, commensalism, and mutualism (see Lewis 1985, Saffo 1992).

Although the study of symbiosis began in the 19th century, little progress was made in our understanding of these associations until the end of the 1900s (Boucher *et al.* 1982, Boucher 1985, Margulis and Fester 1991). It has been speculated that lack of progress in this field, as compared to predation and competition, was in part due to the left-wing politics of the 20th century, though many other reasons have been suggested (see Boucher *et al.* 1982, Bronstein 1994, Sapp 1994). Despite the obvious importance of symbiosis within biology, it still receives limited attention by ecologists and evolutionary biologists. Therefore, many fundamental aspects of symbiosis are still poorly understood, including the evolution of parasite virulence, the origin and stability of mutualisms, and the process of co-evolution. Although our understanding of symbiosis is being greatly improved by studies examining the evolution of microbes in the laboratory (e.g., Bull *et al.* 1991, Turner *et al.* 1998), studies on symbioses in natural systems is still urgently needed. In this thesis, I examine the ecology and evolution of an ancient and highly evolved symbiosis between fungus-growing ants and their fungi.

1.2 THE ECOLOGY AND EVOLUTION OF SYMBIOSIS

1.2.1 Evolutionary stability of mutualism

The question of how mutually beneficial associations between different species evolve has challenged theoreticians for decades (Trivers 1971, Axelrod and Hamilton 1981, Bull and Rice 1991, Doebeli and Knowlton 1998). Based on the selfish-gene theory of evolution (Dawkins 1976), natural selection should not favour one species providing a benefit for another, so organisms should only cooperate if the benefits outweigh the costs. This illustrates the conflict of interest between symbiotic partners, and has led to the view that mutualism is a relationship of reciprocal exploitation more than cooperation (Nowak *et al.* 1994, Maynard Smith and Szathmary 1995, Leigh and Rowell 1995, Herre *et al.* 1999). Because of the inherent conflict of interest, it is predicted that selection should favour symbionts that exploit their partners by taking benefit without providing reward in return. Under such selective pressure, it is not clear how mutualisms remain stable once established (Bull and Rice 1991).

Our understanding of how cooperation evolves and remains stable is largely informed by game theory models. Trivers (1971) was the first to apply the 'Prisoner's Dilemma' game to the evolution of cooperation. He found that although two unrelated partners could benefit by cooperating, they did even better by cheating. The use of an iterated Prisoner's Dilemma game was proposed by Axelrod and Hamilton (1981), in which partners engaged in multiple interactions. Using this scenario, they established that cooperation is favoured when players have multiple interactions and there is an opportunity to punish non-cooperative individuals

(e.g., by not cooperating in future interactions). Recently, Doebeli and Knowlton (1998) modified the iterated Prisoner's Dilemma to allow the amounts exchanged between partners to evolve (perhaps making the model more realistic), and with this modified model they found that mutualisms evolved even more readily. Research on natural systems currently is trying to determine whether, as predicted by these models, mutualism originates and remains stable when species engage in multiple interactions and have an opportunity to punish non-cooperative individuals (e.g., Pellmyr and Huth 1994, Dougatkin 1997, Herre and West 1997).

1.2.2 The symbiotic continuum

Species can interact in neutral, beneficial, or harmful ways, and, as noted above, the term 'symbiosis' encompasses all of these interspecific associations (see 1.1). In addition to representing the different types of symbioses, current theory suggests that symbiosis is a continuum of interactions: from the virulent parasite that rapidly kills its host, on the one end, to the mutually dependent obligatory associations, on the other (Axelrod and Hamilton 1981, Bull and Rice 1991). The costs and benefits of individual symbiotic associations can change on both an ecological and evolutionary scale, moving along this 'continuum'. On an ecological scale, the payoff to each symbiont within established symbioses can depend on both abiotic factors and other interspecific interactions (Gehring and Whitham 1991, Sanders *et al.* 1993, Gastreich 1997). On an evolutionary scale, symbionts can evolve to be less or more exploitative over time. For example, parasites gradually may evolve to become less exploitative, and eventually may become mutualists (Axelrod and Hamilton 1981, Bull and Rice 1991).

The complexity of symbioses, as described above, makes it difficult to delineate associations as either harmful, benign, or beneficial (Cushman and Beattie 1991, Herre and West 1997, Herre *et al.* 1999). It follows from the view that symbioses are continuums and from the inherent conflict of interest between symbionts that understanding how mutualisms evolve and remain stable necessitates an assessment of the costs and benefits to each symbiont. In fact, it has been pointed out that in many mutualisms the presence of a net-benefit to each symbiont has not yet been illustrated (Cushman and Beattie 1991, Bronstein 1994, Bronstein 1998). By extension, attempts to determine which factors influence the costs and benefits of symbiotic relationships are clearly essential to the study of symbioses.

1.2.3 Virulence

Virulence is a measure of the impact a parasite has on its host, with more virulent parasites being more damaging. If, as is currently believed, mutualism is more of a reciprocal exploitation (see 1.2.1), the concept of virulence is central to mutualistic association as well. An understanding of when increases and decreases in exploitation between symbionts will be favoured should provide insight into the evolution of symbioses.

Early theories on the evolution of virulence were heavily influenced by the fact that parasites that kill their hosts die. Therefore, it traditionally has been believed that parasites evolve to be benign and that the more virulent a parasite is, the more poorly adapted it is to its host (e.g., Adler 1964, Dubos 1965, Burnet and Whitten 1972, Alexander 1981). This is based on an inaccurate view of evolution as operating on a population or species level. However, Hamilton (1964) and Williams (1966) clarified that natural selection favours characteristics

that facilitate the passing on of genes that code for those characteristics. Based on this view of evolution, Anderson and May (1981, 1982) point out that selection should favour whatever level of virulence leads to an increase in reproduction in the parasites. A decrease in virulence does not necessarily lead to an increase in reproduction; it typically depends on the mode of transmission. For example, it is well established that parasites that are strictly vertically transmitted (i.e., from parent to offspring) are selected toward lower virulence (May and Anderson 1983, Bull and Rice 1991). By contrast, parasites that are horizontally transmitted (i.e., between unrelated hosts) may be selected toward higher levels of virulence if this facilitates transmission, such as when dispersal occurs through vectors or is waterborne (Ewald 1983, Ewald 1994).

Our current understanding of the evolution of virulence is an over-simplification of the complexity of co-evolution and natural selection as they likely operate on symbionts. For example, some parasites that have high levels of vertical transmission are also known to be virulent (e.g., Kellen and Wills 1962, Vitullo and Merani 1988, Narita *et al.* 1991). In addition, some parasites disperse both vertically and horizontally, and others have very complex life histories (e.g., having several different hosts during their life-cycle).

1.2.4 Co-evolutionary 'arms races' and the Red Queen hypothesis

As pointed out above, selective pressure on symbionts will be toward an increase in net benefit from the association. However, any attempt for a symbiont to increase its net benefit should be counteracted by its host attempting to defend against exploitation. This results in each symbiont evolving in response to the other in a process called co-evolution. Co-

evolution occurs when two or more species evolve in close association, with each species influencing the evolution of the other. In fact, it is suggested that co-evolution only occurs in situations where genetic change occurs in one symbiont as a direct result of genetic change in the other (Futuyma and Slatkin 1983).

The process of co-evolution within symbioses is of obvious importance, as each symbiont undergoes continuous coadaptation in response to its symbiont. On a larger time scale, this process can result in cospeciation or co-cladogenesis between symbionts, with each symbiont lineage forming new species in synchrony with each other. Not all co-evolutionary interactions lead to cospeciation since deviations to new symbionts may occur through host switching or a symbiont returning to its ancestral free-living form. The processes of coadaptation, cospeciation, and host switching are fundamental aspects of the evolution of symbiosis.

When symbionts co-evolve in a series of escalating adaptations and counter-adaptations, the symbionts are engaged in what is referred to as a 'co-evolutionary arms race'. That is, each symbiont continuously adapts to the selective pressure exerted on it from the other, with an increase in the extent of the offensive or defensive components utilized by each. The locking of symbionts into such an escalating co-evolutionary arms race has been called the 'Red Queen' theory. This term was proposed by Van Valen (1973) in reference to Lewis Carroll's *Through the Looking Glass*: Alice and the Red Queen had to continuously run faster and faster to stay in the same place.

The Red Queen was later adapted by Jaenike (1978) into a hypothesis which attempts to explain the selective force that maintains the dominance of sexual reproduction. It is commonly recognized that asexual organisms can propagate their genes twice as quickly as their sexual conspecifics (Williams 1975, Maynard Smith 1978). Considering the competitive advantages of asexual reproduction, some selective force must favour sexual reproduction. Many theories have been generated to explain the dominance of sex, however, one of the most promising is the Red Queen hypothesis, which suggests that sexual reproduction is an advantage for organisms in their co-evolutionary arms race with parasites. This hypothesis is based on the belief that parasites are able to rapidly adapt to genetically homogenous hosts. Only through obtaining new or novel resistant genotypes through the process of sexual recombination can hosts stay ahead of their parasites in their co-evolutionary arms race.

In contrast, Law and Lewis (1983) suggest that within certain mutualisms there should be selection toward asexual reproduction. This idea was proposed specifically for endosymbionts (called 'inhabitants') and their hosts (called 'exhabitants', after Starr 1975), as the exhabitant is thought to provide a protective and stable environment for the inhabitant that lives within it. However, if an inhabitant undergoes sexual reproduction, this can result in innovation that leads toward incompatibility with its exhabitant. Law and Lewis (1983) tested this theory by conducting a meta-analysis and found, as predicted, that endosymbionts had higher rates of asexual reproduction than their free-living counterparts.

Co-evolutionary arms races are still poorly understood. For example, it currently is not clear why some associations lead to high degrees of cospeciation and others experience frequent host switches. Obviously, the transmission of symbionts will affect the amount of cospeciation or host-switching, with symbionts that have greater ability to disperse without their host leading to more host switches, and those that depend on their symbiont for transmission having higher degrees of cospeciation. In addition, the theory that sexual reproduction is maintained because it is advantageous for hosts in their co-evolutionary arms races with parasites still is not well established through empirical data (Ebert and Hamilton 1996). Finally, it is perhaps surprising that the arms race between inhabitants and exhabitants results in a decrease in sexual reproduction, particularly when mutualisms are regarded as mutual exploitation, and sexual reproduction is thought to be an important mechanism by which organisms avoid being exploited by their symbionts.

1.2.5 Parasites of mutualisms

Parasites of mutualisms have been a major focus of study for decades (Boucher *et al.* 1982, Mainero and Martinez del Rio 1985). However, the vast majority of this attention has been on 'cheaters' (e.g., Morris 1996, Pellmyr *et al.* 1996, Addicott 1996, Bao and Addicott 1998). Cheaters are taxa that are closely related to one symbiont, but instead of cooperating, they obtain benefit without reciprocating. This attention has been based on the interest in the evolutionary stability of mutualisms (see 1.2.1). Another likely reason that biologists have focused on cheaters is that these organisms are already highly co-evolved within their particular mutualism, and thus are thought to be well suited to take advantage of them.

In contrast, unrelated parasites of mutualisms have received very little scientific attention. It is possible that mutualisms are inherently resistant to exploitation by unrelated taxa, and thus the lack of scientific attention is just a reflection of their limited biological importance. However, the more likely explanation is that unrelated parasites of mutualisms have been overlooked by biologists, perhaps because of the greater interest in cheaters.

Mutualisms are ubiquitous and abundant, involving the exchange of valuable resources (Boucher 1985, Ahmadjian and Paracer 1986). Thus mutualisms should be promising resource-rich hosts for parasites. In addition, if the inhabitants within mutualisms do evolve toward being asexual, as predicted by Law and Lewis (1983), then the Red Queen hypothesis predicts higher parasite pressure (see 1.2.4). I suggest that mutualisms, like all biological organisms themselves, are not resistant to unrelated parasites, but rather these parasites are important and greatly overlooked components of mutualistic associations. In this thesis, I will examine the importance of unrelated parasites in the mutualism between attine ants and their fungal cultivars.

1.3 MUTUALISM BETWEEN FUNGUS-GROWING ANTS AND THEIR FUNGI

1.3.1 Introduction

The obligate mutualism between fungus-growing ants and their fungi is a textbook example of symbiosis. The ants cultivate the fungus, providing it with substrate on which to grow, protection from competitors, and dispersal to new colonies. In exchange, the fungus serves as the ants' main food source. In fact, this is one of only a few examples of organisms

growing their own food, and thus has been described as a major breakthrough in animal evolution (Wilson 1986). Fungal cultivation by fungus-growing ants is ancient, likely having originated more than 50-65 million years ago (Wilson 1971, Mueller *et al.* submitted). Fungus-growing ants are only New World in distribution, indicating that the origin (likely in the tropical rainforests of Amazon basin) and subsequent radiation throughout the neotropics occurred after the separation of South America from Africa in the mid- to late Cretaceous (Weber 1972).

The evolution of agriculture in fungus-growing ants has led to their dominance in the neotropics. They have a vast geographic distribution, occurring as far north as New Jersey (40° north latitude) and as far South as central Argentina (44° south latitude). They are both abundant and diverse throughout most of this range, especially in wet tropical regions. However, their success is best illustrated by the phylogenetically derived group referred to as the leaf-cutters. Leaf-cutting ants have one of the most complex social systems of all insects (Wilson 1980a, Wilson 1980b). They also play an important role in the functioning of neotropical ecosystems, facilitating nutrient cycling and stimulating new plant and root growth (Haines 1978, Hölldobler and Wilson 1990). In addition, they are considered the dominant herbivore in the New World (Hölldobler and Wilson 1990), cutting perhaps as much as 17% of the vegetation produced in tropical forests (Cherrett 1986). Some leaf-cutting ant species do exceptionally well in disturbed habitats, as is the case in human agriculture. This has resulted in these ants becoming one of the most destructive pests of agriculture in Central and South America, doing over a billion dollars worth of damage per year (Hölldobler and Wilson 1990).

Fungus-growing ants belong to the monophyletic tribe Attini (Hymenoptera: Formicidae: subfamily Myrmicinae), which is composed of 12 genera and approximately 210 described species (Table 1.1, Schultz and Meier 1995). The 5 most phylogenetically derived genera form a clade called the 'higher attines', while the remaining 7 genera, with approximately half of the species diversity, compose the 'lower attines'. Although all attine ants are referred to as fungus-growers, the two most phylogenetically derived genera, *Acromyrmex* and *Atta*, are more commonly known as leaf-cutting ants. The lower attines typically use dead vegetative debris as well as insect feces and corpses to manure their gardens, while the phylogenetically basal genera of the higher attines (e.g., *Trachymyrmex* and *Sericomyrmex*) typically use dead vegetable matter (Table 1.1). As mentioned above, the leaf-cutting ants use mostly fresh leaves and flowers.

Table 1.1 Genera of fungus-growing ants organized by evolutionary sequence from the most phylogenetically basal to the most phylogenetically derived (Based on Schultz and Meier 1995 and Wetterer *et al.* 1998). The ecological characteristics of each genus are outlined, illustrating the evolutionary trend toward increasingly complex fungiculture. The genus *Pseudoatta* is not included, as it is only known as a worker-less social parasite.

Attini genera	Ant Groupings	Symbiont Clade	Size of Workers	Worker Polymorphism	Colony size	Garden substrate
<i>Myrmicocrypta</i>	Lower attine	G3	Small	Monomorphic	Small to medium	Insect corpses
<i>Mycocepurus</i>	Lower attine	G3	Small	Monomorphic	Small	Insect feces
<i>Apterostigma</i>	Lower attine	G2	Small to medium	Monomorphic	Small	Insect feces and woody matter
<i>Mycetarotes</i>	Lower attine	Unknown	Small	Monomorphic	Small	Unknown
<i>Mycetosoritis</i>	Lower attine	G3	Small	Monomorphic	Small	Dead vegetative matter
<i>Cyphomyrmex</i>	Lower attine	G3	Small	Monomorphic	Medium	Insect feces, corpses
<i>Mycetophylax</i>	Lower attine	G3	Small	Monomorphic	Small	Dead grass

Table 1.1, continued:

<i>Sericomyrmex</i>	Higher attine	G1	Medium	Monomorphic	Medium	Dead vegetative matter
<i>Trachymyrmex</i>	Higher attine	G1	Medium	Slightly polymorphic	Medium	Dead vegetative matter
<i>Acromyrmex</i>	Higher attine & Leaf-cutter	G1	Medium to Large	Polymorphic	Large	Fresh leaves and flowers
<i>Atta</i>	Higher attine & Leaf-cutter	G1	Large	Strongly polymorphic	Very Large	Fresh leaves and flowers

Human interest in attine ants, especially the conspicuous leaf-cutters, is likely as old as our geographical overlap with them. Although there is no direct evidence of thousands of years of interaction between leaf-cutting ants and humans, they are mentioned in the Popul Vuh, a creation myth of the Mayan civilization from 300-900 AD, and there are extensive reports made by early European colonizers in South America describing these ants as the 'scourge' of the New World (see Weber 1966, Weber 1972, Hölldobler and Wilson 1990). Linnaeus was the first biologist to be interested in fungus-growing ants, listing several species from the Attini in his classic book *Systema Naturae* in 1758. Later, in 1804, Fabricius described the genus *Atta*, which is the type for the tribe. Much of the generic classification was originated by Mayr in the 1860s. Extensive observations on the behaviour of these ants were made by Bates and described in his 1863 book, *The Naturalist on the River Amazon*. However, Thomas Belt (1874) was the first to discover that the conspicuous trails of ants that carry leaves deep underground were not using this vegetative material as food, but instead as manure to grow fungal gardens on which to feed. Möller (1893) followed up Belt's work with an extensive mycological study of this mutualism, including the first observations of ants consuming the fungus. Extensive investigation of fungus-growing ants was continued into the 20th century, resulting in the publication of thousands of scientific articles (Cherrett 1986, U.G. Mueller personal communication).

1.3.2 Evolution of fungus-growing in attine ants

The majority of the research conducted on fungus-growing ants has concentrated on the foraging behaviour of the leaf-cutters. Obviously this trend has been driven by the agricultural pest status of these ants. Nevertheless, recent studies on fungus-growing ants

and their fungi have resulted in this mutualism becoming a model system for the study of symbioses (e.g., Chapela *et al.* 1994, North *et al.* 1997, Mueller *et al.* 1998).

Evolution of the Ants

Little progress on the evolutionary history of fungus-growing ants was made over most of the 20th century; however, two important phylogenetic studies were published in the 1990s. The most significant of these was done by Schultz and Meier (1995), who conducted an extensive study of larval morphology, including over 50 taxa and using 44 characters. The other study, by Wetterer *et al.* (1998), generated a phylogeny of the Attini based on DNA sequences. Although this latter study is limited in its taxonomic sampling, it does provide further support for the phylogeny reconstructed by Schultz and Meier.

These studies strongly support the monophyly of fungus-growing ants, indicating that fungal cultivation in the ants has a single evolutionary origin. In addition, they reconstruct the phylogenetic history within the Attini, providing a well supported evolutionary sequence for the attine genera (Figure 1.1). This provides the framework for an understanding of the evolution of these ants once fungal cultivation originated. For example, prior to this work, it was assumed that the genus *Cyphomyrmex* was the most phylogenetically basal because some species cultivate yeasts (e.g., Weber 1966, 1972, Hölldobler and Wilson 1990). However, Schultz and Meier (1995) establish that this genus is derived within the lower attine clade.

Schultz and Meier (1995) also provide insight into the origin of fungiculture in these ants. To understand how this behaviour evolved, knowledge of the life history of the direct ancestors of these ants prior to their cultivating fungi is needed (Mueller *et al.* submitted). Clearly direct study of this is not possible, so the best alternative is to examine the biology of the closest living relative to the fungus-growers as well as the most basal genus within the Attini. In their analyses, Schultz and Meier (1995) included all 12 myrmicine species that have been proposed to be the sister group to the fungus-growing ants. Their results support the myrmicine genera *Blepharidatta* and *Wasmannia* being the sister groups to the fungus-growing ants. In addition, their phylogeny places the genus *Myrmicocrypta* as the most basal genus within the Attini. Future work focusing on these ant genera should lead to new insight into the evolutionary origin of this fascinating mutualism (see Mueller *et al.* submitted).

Evolution of the fungi

Understanding of this complex mutualism has been greatly hindered by the complete lack of information regarding the evolutionary history and taxonomic placement of the fungal cultivars. The traditional methods for fungal taxonomy and systematics depend on the morphology of fruiting structures, however, the fungi cultivated by attine ants do not produce these sexual structures in association with the ants nor in pure culture (Möller 1893, Hervey *et al.* 1977, Muchovej *et al.* 1991). The recent development of molecular phylogenetic techniques has allowed the evolutionary history of these fungi to be reconstructed and their taxonomic placement established (Chapela *et al.* 1994, Mueller *et al.* 1998).

Chapela *et al.* (1994) examined the evolutionary history of the fungi cultivated by attine ants, using cladistic analyses of the nuclear 28S ribosomal DNA. Specifically, they reconstructed the phylogeny for fungi cultivated by 19 species of attine ants. They establish that the fungi cultivated by fungus-growing ants are polyphyletic. Most of the fungi belong to the family Lepiotaceae, in the tribe Leucocopriini (Agaricales: Basidiomycota), a group of mostly tropical species that are specialized litter decomposers (Dennis 1952, Singer 1986). In addition, they establish that some species of ants in the Attini genus *Apterostigma* cultivate fungi in the family Tricholomataceae (Agaricales: Basidiomycota). These cultivars appear to be closely related to the wood decomposing fungi in the genus *Gerronema* (Moncalvo *et al.* 2000). A subsequent study by Mueller *et al.* (1998) (see below for details) indicates that, in fact, there are at least 4 distinct clades of Leucocoprineous fungi that are cultivated by fungus-growing ants.

Ant-fungal co-evolution

Möller (1893), in his extensive mycological study of this mutualism, found the first signs of co-evolution between the ants and their fungal cultivars. Specifically, he discovered that the fungi cultivated by the higher attines produce specialized swellings, later termed gongylidia, at the tips of their hyphae. He observed that it is these structures that are consumed by the ants. Later work established that gongylidia provide the optimum nutrients for the ants, being high in carbohydrates, free and protein-bound amino acids, and lipids (Martin *et al.* 1969, Quinlan and Cherrett 1979).

Based on this discovery by Möller (1893) and on his own observations of attine ants, Wheeler (1910) suggests that there has been a progression toward more complex agriculture in attine ants and their fungal cultivars. He points out that the more derived genera have larger worker size, and orders of magnitude larger colony size, than the basal genera (Table 1.1). In addition, the social system is increasingly more complex in the derived genera. The lower attines are monomorphic in worker size, while the more phylogenetically derived genera, especially *Acromyrmex* and *Atta*, are strongly polymorphic in worker size. There is also a large difference in the substrates utilized to manure the fungus (Table 1.1). Hölldobler and Wilson (1990), among others, have also noted this evolutionary trend.

Using the ant phylogeny reconstructed by Schultz and Meier (1995) and their own phylogeny of the fungal cultivars, Chapela *et al.* (1994) examined the co-evolution between the ants and their fungi. They sequenced the 28S rDNA gene in this study, which is a relatively conserved locus within fungal genomes. This allowed the co-evolution between the ants and their fungi to be examined between, but not within, genera. They establish that the lower and higher attines each cultivate their own distinct clade of fungi (the G3 and G1 clades, respectively). A third clade of symbionts, as mentioned above, is cultivated by species in the genus *Apterostigma* (the G2 cultivars). Their reconstruction of the evolutionary history of attine ant cultivated fungi also suggests that the higher attines have been clonally propagating their fungi (G1 clade) for millions of years.

Mueller *et al.* (1998) conducted an elaborate study examining the evolutionary history of fungi cultivated by lower attines, specifically members of the G3 clade (from Chapela *et al.*

1994). They sampled extensively across the 7 genera of the lower attines and a wide geographic region, obtaining more than 553 isolates of fungi. In addition, they collected and cultured free-living members of the *Leucocoprinini* that were suspected to be the closest free-living relatives to the cultivars of the lower attines (n=309). Then, using population genetic techniques and sequencing of conserved genes, they examined the co-evolution between the ants and fungi, both within and between ant genera. They found that within the same population, distantly related ant species can cultivate the same fungal clone. In addition, the same ant species can cultivate distantly related cultivars. They conclude that ants can acquire new cultivars from the closely related free-living representatives of *Leucocoprinus*. Therefore, the fungi cultivated by the lower attines have not been strictly clonally propagated for millions of years, as is apparently the case with the higher attines (see above).

Summary

The ancient association between fungus-growing ants and their fungi has resulted in a complex evolutionary history between these mutualists. With the origin of the unique ability to cultivate fungi, the Attini have diversified and radiated throughout the neotropics (Schultz and Meier 1995). Evolution within the fungal cultivars and co-evolution between the fungi and the ants is even more complex. In the lower attines, the history of association appears to be shaped by frequent host switching, based on the ants re-acquiring cultivars from free-living *Leucocoprineous* fungi. In contrast, the highly derived ants cultivate highly derived fungi, which they apparently have been clonally propagating for millions of years (Chapela *et al.* 1994). The evolutionary transition from cultivating genetically diverse and sexually reproducing cultivars, as in the lower attines, to clonally propagating fungi, in the higher

attines, is fascinating. As pointed out above, genetically homogenous asexually reproducing organisms are subject to serious parasite pressure (1.2.4). Human agriculture has experienced serious parasite problems as a result of growing genetically homogenous cultivars, indicating that the ants' ability to clonally propagate fungi for millions of years is surprising.

1.3.3 Ecology of fungus-growing in attine ants

Introduction

Although significant progress toward understanding the evolution of this ant-fungal symbiosis has been made (1.3.2), the actual process of fungal cultivation is still relatively poorly understood. It is obvious that the ants cultivate and care for the fungi, but it is currently not clear how this is done successfully. In addition, the specific interactions and costs and benefits of this association by and large are unexplored. Most of the research examining the mechanisms of fungiculture in attine ants has been done on the leaf-cutters, so most of my discussion on fungal gardening will be focused on this group of ants.

Manuring and promoting the growth of the garden

Fungiculture begins with the collection and addition of substrate to the garden. The ants begin the decomposition process by licking and then masticating the leaf material, breaking it into pieces as small as 1-2 millimeters. The resulting pulp is sometimes applied with a fecal droplet, which is believed to add important enzymes to help the fungus break down the material (see below). The chewed up pulp is then brought to the top of the garden and worked into position. At this stage, fungal inoculum from older parts of the garden is

brought up and planted onto the surface of this fresh substrate. With continuous tending by the ants, the fungus grows quickly, potentially covering the whole surface in a few days. Most of the garden tending activities are conducted by the minima workers (smallest size workers), whose size is believed to be optimal for tending the garden (Bass and Cherrett 1996). It takes approximately 6 weeks for the vegetative material to be completely broken down by the fungus, at which point this used-up material is removed from the bottom of the garden and placed in the dump.

Obviously, there is much more involved in cultivating fungal gardens than planting new substrate on top and removing the old, spent material from the bottom. In fact, throughout this process workers are continuously promoting the growth of the fungus. The ants move proteolytic enzymes around the garden, from locations of high concentration to low ones (Martin 1970, Martin and Martin 1970). Interestingly, these enzymes are apparently produced by the fungus, and just applied by the ants (Boyd and Martin 1975). In addition, Bass and Cherrett (1996) provide evidence that the ants mechanically damage the hyphae to stimulate their growth, perhaps in effect pruning them. The ants also optimize the climatic conditions for the fungus by opening or closing tunnels to the surface, thus regulating the temperature and humidity within the colony.

Dispersing the fungus

In addition to manuring and promoting the growth of the fungal cultivars, the ants disperse the fungus to new colonies. Prior to the nuptial flight, gynes (female alates or reproductives) collect a small ball of fungus from their parent colony. The new queens store the fungus in

their infrabuccal pocket, which is located inside their mouthparts, and carry inocula during the nuptial flights. After digging a claustral chamber the queens spit-up the fungus and begin tending it underground. Thus, the fungus is primarily dispersed vertically (or clonally), from parent to offspring colonies. Species of the lower attines that cultivate Leucocoprineous cultivars (G3 clade from Chapela et al 1994), apparently re-acquire new cultivars from free-living stock (Mueller *et al.* 1998), but it is not clear how frequently this occurs.

Defending the garden

Cultivating fungi requires complex behavioural and physiological adaptations (such as those outlined above); however, it is the ability to maintain healthy fungal gardens that is usually described as the most important (Weber 1966, 1972, Hölldobler and Wilson 1990) because the garden is continuously inoculated with bacteria and fungi on the substrate added to the garden. In fact, most of these microbes are superior competitors to the fungi cultivated by the ants, and they rapidly over-grow the fungal gardens in the absence of the ants (Möller 1893, Weber 1956, 1957, 1966). Despite the obvious necessity of protecting the garden from 'alien' microbes to the success of this amazing mutualism, I believe that this is the least understood aspect of this symbiosis, despite what is indicated in the literature (Weber 1972, Hölldobler and Wilson 1990, North *et al.* 1997).

Neil Weber, who has studied fungus-growing ants for almost five decades, argues in his 1972 monograph on attine ants that the primary mechanism for maintaining healthy fungal gardens involves providing optimum growing conditions for the fungi. He suggests that this process allows the ants' cultivars to out-compete other microbes that are present in the garden.

Specifically, he argues that one of the primary means of defense against 'alien' microbes is the movement by the ants of digestive enzymes into needed locations in the garden (see above). However, there is currently no evidence that the promotion of their own cultivars suppresses growth of and invasion by alien microbes. In fact, alien fungi would be expected to benefit from the presence of these enzymes in the same way that the cultivars of the ants do. Furthermore, removal of the ants from the garden leads to rapid over-growth of the fungus, often in less than a day or two. It is unlikely that this outcome is due primarily to rapid deterioration of the garden growing conditions. I suggest, contrary to Weber, that the ability of the ants to suppress the growth, or even eliminate the presence, of 'alien' microbes is likely the mechanism preventing the over-growth of the garden.

One of the primary mechanisms thought to be used by the ants to suppress alien microbes is antibiotic production in their metapleural glands (e.g., Hölldobler and Wilson 1990, Chapela *et al.* 1994, North *et al.* 1997, Mueller *et al.* 1999). The evidence to support this assumption is the presence of compounds with antimicrobial properties within the gland (Maschwitz *et al.* 1970, Schildknecht and Koob 1970, Schildknecht and Koob 1971, Nascimento *et al.* 1996). However, the presence of metapleural glands is symplesiomorphic (shared-ancestral) in all ants, and the ancestral role of the gland appears to be for producing antibiotics to protect the ants from entomopathogenic microbes (Maschwitz *et al.* 1970, Schildknecht and Koob 1971, Hölldobler and Engel-Siegel 1984, Beattie *et al.* 1985, Beattie *et al.* 1986, Veal *et al.* 1992). Although it is possible that the metapleural gland evolved in the Attini to help maintain the health of the garden, Weber (1982) has pointed out that establishing this fact would necessitate demonstrating that the metapleural gland compounds are present within the

garden at a sufficient level to suppress microbes. This has not been demonstrated. Also, the metapleural gland is located on the dorsal surface of ants, which is not a convenient location for applying compounds to the garden. Finally, the bioassays that establish the presence of fungistatic and bacteriostatic compounds in fungus-growing ants are questionable. For example, some studies have found little or no antibiotic activity (Nascimento *et al.* 1996, Bot *et al.* in preparation). In addition, the bioassays that have shown activity have not been conducted against relevant microbes (i.e., ones that actually would invade and grow in fungal-gardens). Considering the current lack of evidence, it cannot be assumed that the metapleural gland has any role in protecting the gardens of fungus-growing ants.

Fungus-growing ants also are assumed to defend the garden through the physical removal of 'alien' microbes by workers. Weber (1957), in a short abstract, was the first to suggest that the ants 'weed' the garden of alien microbes. Despite the fact that no empirical evidence is present in this abstract, and that Weber (1972) later indicated that he "no longer considered [weeding] important", this is commonly cited as one of the main defense mechanism of the ants (e.g., Hölldobler and Wilson 1990, Chapela *et al.* 1994, North *et al.* 1997). It is likely that the ants physically remove 'diseased' parts of the garden, however, further work is needed to establish the importance of this weeding behaviour in maintaining healthy fungal gardens.

Several other alternative methods for maintaining the health of the fungal gardens have been proposed but have received significantly less attention. One possible method is the fungus itself producing antibiotics that help protect it from competitors or parasites. Several studies

have addressed this possibility with mixed results. Martin *et al.* (1969), Weber (1972), and Stradling and Powell (1986) found no antifungal activity in the symbiont, while both Harvey and Nair (1979) and Wang *et al.* (1999) found some antibiotic activity. In addition, the pH of the garden is relatively low, and is thought to be a means of suppressing contaminants, particularly bacteria (Powell and Stradling 1986).

1.3.4 Alien microbes in ant fungal gardens

Since the discovery over a century ago that attine ants cultivate fungi, biologists have assumed that ants maintain their mutualist fungus in pure 'monocultures', preventing other microbes and parasites from occurring in the garden (see for example, Weber 1966, Martin *et al.* 1969, Weber 1972, Fisher *et al.* 1996, North *et al.* 1997). However, the assumption that the gardens are "monocultures" has not been rigorously tested or even theoretically scrutinized.

Craven *et al.* (1970) used dilution plating techniques and scanning electron microscopy to examine gardens for non-mutualistic microbes. Specifically, they studied a colony of *Acromyrmex octospinosus* and *Atta cephalotes*, two common leaf-cutting ant species, and identified the presence of an abundance of yeasts. However, they only sampled from a single garden of each species and did not identify the microbes isolated. Also, it is not clear whether these colonies were recently collected from the field, or were maintained under laboratory conditions for a significant period before the isolations were conducted. A recent study also isolated yeasts in leaf-cutting ant fungal gardens (Carreiro *et al.* 1997). However, the gardens they isolated from had been maintained in the laboratory for 6 months prior to

the study, suggesting the possibility that the yeasts were present only because the laboratory provides less optimal growth conditions for the garden.

The only examination of non-mutualistic filamentous fungi within ant gardens was conducted by Fisher *et al.* (1996). They sampled from 3 *A. cephalotes* colonies collected in Trinidad. Only one of these colonies was sampled in the field; the remaining two were maintained in laboratory for months prior to sampling. They isolated mostly well known endophytic and epiphytic fungi, which they admit were brought into the garden in the leaf material fed to colonies in the laboratory.

1.4 THESIS OBJECTIVES

The ancient and highly evolved mutualism between fungus-growing ants and their fungi is a model system in the study of symbiosis (Weber 1972, Hölldobler and Wilson 1990, Chapela *et al.* 1994, North *et al.* 1997, Mueller *et al.* 1998). The ants and fungi are mutually dependent, so the maintenance of stable fungal cultures is critical to the survival of both organisms. In fact, this mutualism is currently thought to be so successful that the fungus-growing ants are believed to maintain their gardens free of parasites in axenic 'monocultures'. The ants are believed to eliminate 'alien' microbes by weeding, applying antimicrobial compounds to the garden (from their metapleural glands), and moving around proteolytic enzymes within the garden (1.3.3). However, the absence of microbial parasites within the gardens has neither been established nor adequately examined, and even the mechanisms for protecting the garden from microbes are being challenged (1.3.3).

In this thesis, I conduct the first extensive examination of microbial parasites of gardens of attine ants. My 'a priori' hypothesis is that the gardens of fungus-growing ants are host to highly specialized and potentially virulent parasites. This is based on theoretical predictions derived from the study of evolution of parasites. First, all organisms are thought to be host to parasites. More importantly, the ants' fungal cultivars are genetically homogenous because they are clonally propagated from one generation to the next. The Red Queen hypothesis predicts that these ancient asexual clones should face higher parasite pressure. In addition, based on the only well studied analogous system, human agriculture, it is well established that clonally propagated cultivars result in serious pest and pathogen problems. In fact, the vulnerability of human grown monocultures to parasites has even been used as partial evidence of the Red Queen hypothesis (e.g., Ebert and Hamilton 1996).

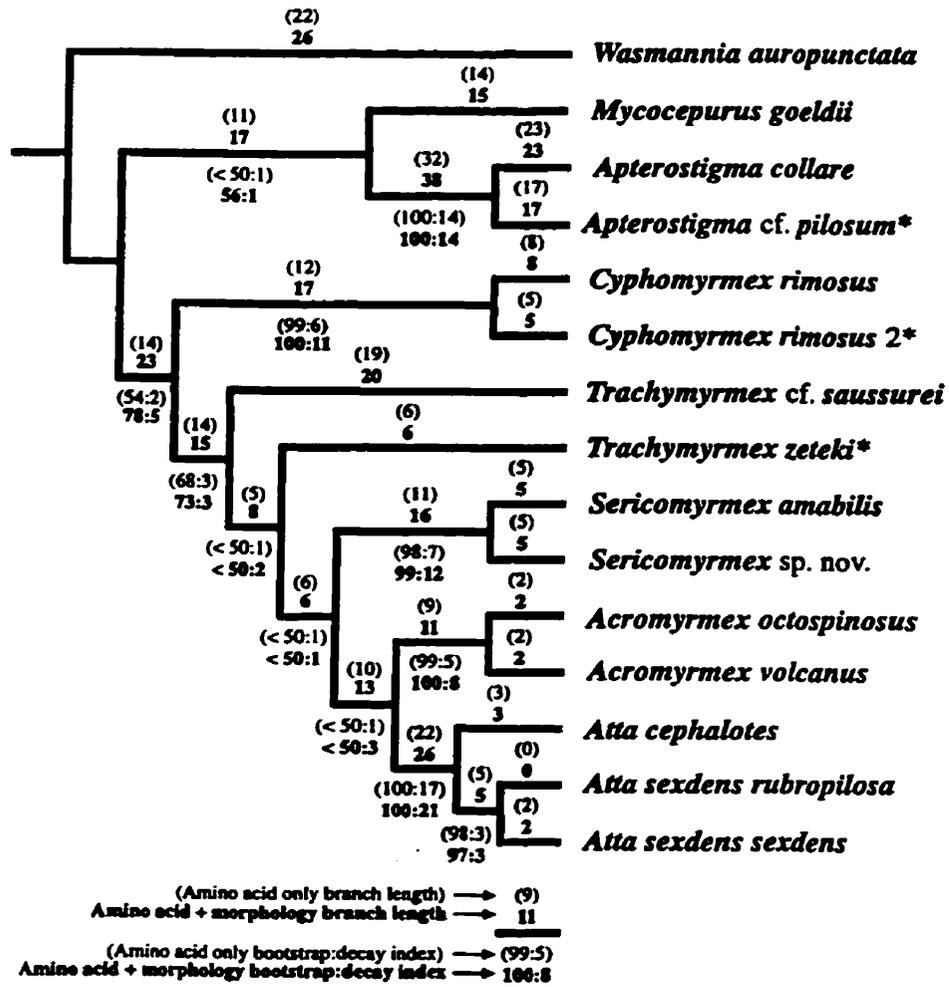
The majority of studies examining parasites of mutualisms study 'cheaters', but I suggest that the presence of unrelated parasites may be an important but greatly overlooked component of symbiosis (see 1.2.5). The alternative hypothesis is that mutualisms are inherently resistant to exploitation, partially because of their long co-evolutionary history. This assumption, however, does not account for the possibility that parasites have also been a component of this co-evolutionary history, perhaps forming additional assemblages across the history of such associations. My examination of unrelated parasites within the mutualism between fungus-growing ants and their fungi is a good test of the prediction that these largely unexamined components of mutualism are important. Thus, an additional objective of my thesis is to examine the importance and possible co-evolution of unrelated parasites within this mutualism.

As discussed above, the ants and their fungi are mutually dependent, therefore the maintenance of stable fungal monocultures in the presence of weeds or parasites is critical to the survival of both organisms. In fact, the gardens of fungus-growing ants are continuously inoculated with competitively superior microbes that are ubiquitous in the substrate the ants use to manure their fungal cultivars. Thus, the success of this mutualism is solely dependent on ants and their fungi being able to deal with these alien microbes. Despite the fact that this is of fundamental importance to the success of this well studied mutualism, current explanations of how it is accomplished are seriously lacking (see section 1.3.3). In addition, the discovery of a virulent specialized parasite of the garden (see section 2.0 and section 3.0) makes the interaction of the ants and their fungi with alien microbes even more central to this mutualism.

In the second section of my thesis, I examine a possible new method employed by the ants for maintaining the health of their fungal gardens. First, I discuss my discovery that fungus-growing ants carry a filamentous bacterium (actinomycete: *Streptomyces*) on their exoskeleton (see section 3.0). As these bacteria are well known for the production of antimicrobial metabolites (i.e. a majority of antibiotics used by humans are produced by bacteria in the genus *Streptomyces*), I examine the hypothesis that fungus-growing ants have evolved a symbiotic association with an actinomycete that produces antibiotics to maintain the health of their gardens. This hypothesis is examined by testing the ant associated microbes for the production of antimicrobial compounds and making further examinations of its symbiotic association with attine ants. Thus, the final objective of my thesis is to examine the possibility that unrelated organisms play a beneficial role in this mutualism. This

investigation is a further challenge to the assumption that mutualisms occur in isolation and are not mediated by additional organisms.

Figure 1.1 The phylogenetic relationship among attine ants based on both larval morphology and amino acid sequence of mitochondrial DNA. (Source: “Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology” by Wetterer *et al.* 1998, in *Molecular Phylogenetics and Evolution*, Volume 9(1), 42-47, copyright © 1998 by Academic Press, reproduced by permission of the publisher)



2.0 THE AGRICULTURAL PATHOLOGY OF ANT FUNGUS GARDENS

(Previously published in *Proceedings of the National Academy of Sciences, USA* 96:7998-8002, with U.G. Mueller and D. Malloch)

2.1 ABSTRACT

Gardens of fungus-growing ants (Formicidae: Attini) traditionally have been thought to be free of microbial parasites, with the fungal mutualist maintained in nearly pure “monocultures”. I conducted extensive isolations of “alien” (non-mutualistic) fungi from ant gardens of a phylogenetically representative collection of attine ants. Contrary to the long-standing assumption that gardens are maintained free of microbial pathogens and parasites, they are in fact host to specialized parasites that are only known from attine gardens and that are found in most attine nests. These specialized garden parasites, belonging to the microfungus genus *Escovopsis* (Ascomycota: anamorphic Hypocreales), are horizontally transmitted between colonies. Consistent with theory of virulence evolution under this mode of pathogen transmission, *Escovopsis* is highly virulent and has the potential for rapid devastation of ant gardens, leading to colony mortality. The specialized parasite *Escovopsis* is more prevalent in gardens of the more derived ant lineages than in gardens of the more “primitive” (basal) ant lineages. Because fungal cultivars of derived attine lineages are asexual clones of apparently ancient origin, while cultivars of primitive ant lineages were domesticated relatively recently from free-living sexual stocks, the increased virulence of pathogens associated with ancient asexual cultivars suggests an evolutionary cost to cultivar clonality, perhaps resulting from slower evolutionary rates of cultivars in the co-evolutionary race with their pathogens.

2.2. INTRODUCTION

Fungus gardening by ants originated around 50 million years ago (Wilson 1971). The subsequent evolutionary history of this obligatory mutualism has resulted in complex associations among the ants and their fungal symbionts. The attines comprise about 200 described species in 12 genera that are obligately dependent on fungiculture for food. While attine ants derived from a single ancestor (Schultz and Meier 1995, Wetterer *et al.* 1998), extant species cultivate multiple, phylogenetically distant lineages of fungi, most of them belonging to the family Lepiotaceae (Agaricales: Basidiomycota) (Chapela *et al.* 1994, Mueller *et al.* 1998). The fungal cultivars, serving as the primary food source for the ants, are carefully manured by the ants with plant substrate, insect frass, or seeds. Foundress queens propagate the fungus clonally by carrying inocula in their mouths during their nuptial flight to establish new colonies (von Ihering 1898, Huber 1905). While at least some “lower” attines (phylogenetically basal lineages) propagate cultivars that were recently domesticated from free-living populations of Lepiotaceae (Mueller *et al.* 1998), the “higher” attines (a derived monophyletic clade that includes the leaf-cutting ants) are thought to propagate ancient clones, likely several million years old (Chapela *et al.* 1994).

Among the attines, ants in the two genera of leaf-cutting ants (*Acromyrmex* and *Atta*) exhibit the most complex fungicultural systems. Fungiculture among *Atta* supports colony populations in the millions of workers and explains why leafcutters are the dominant herbivore in the Neotropics (Hölldobler and Wilson 1990). Colonies of *Atta* spp. can survive for extended periods, often living for 8-10 years or more after initial nest founding (Weber 1966). Foundress queens of these species establish new colonies by digging chambers in the

soil, expelling the fungal pellet that they bring from their natal nest, and initiating the cultivation of their own gardens, which are started using fecal material provided by the queen. Nest-founding occurs within a claustral chamber that remains closed until the first brood is reared, at which point the new workers begin foraging for leaf material outside the initial chamber.

Fungus-growing ants are thought capable of cultivating their fungus in axenic (single species) “monocultures”, despite continuous exposure to the competitively advantaged microbes already present in the vegetative material that is added to the garden. The ability of the ants to maintain the fungus garden in monocultures was first proposed by Möller (1893) over a century ago in the first mycological study of attine gardens. Further support for this hypothesis has been derived from later observations that ants weed out ‘alien’ microbes, produce anti-microbial chemicals, and increase the competitive advantage of the mutualist through application of proteolytic enzymes (Weber 1957, Schildknecht and Koob 1970, Martin 1987, Bass and Cherrett 1994). Although these behaviors are important for successful fungal cultivation, there is no empirical evidence indicating that they result in pure garden cultures. The few studies sampling microbes from attine-gardens have been unable to identify the presence of microbes capable of circumventing the defenses of the ants, and thus persisting in the garden as specialized parasites (Craven *et al.* 1970, Fisher *et al.* 1996, Carreiro *et al.* 1997). These studies were limited in scope, with extremely small sample sizes (one, three, and ten nests, respectively), included only one or two attine species, and were primarily conducted on colonies maintained under laboratory conditions for months prior to sampling (Craven *et al.* 1970, Fisher *et al.* 1996, Carreiro *et al.* 1997).

Although several studies identified adventitious extraneous microbes that are brought into the gardens on vegetative foraging material (Fisher *et al.* 1996, Carreiro *et al.* 1997), the presence of specialized parasites has neither been established nor adequately studied. The longstanding assumption that ant fungal gardens are free of significant pathogenic pressure is surprising, because it contradicts some fundamental theories of the evolution of parasitism. Specifically, parasites are thought to be a selective force maintaining genetic diversity and hence sexual reproduction, typically referred to as the Red Queen hypothesis for the evolution of sexuality (Jaenike 1978, Hamilton 1980). In fact, the devastating impact of parasites on human cultivated monocultures is used as partial evidence in support of this theory (cf. Seger and Hamilton 1988, Ebert and Hamilton 1996). To resolve the conflict between the theoretical prediction that parasites should exist in the clonal attine fungicultural systems, and the widespread yet untested belief that ants maintain their gardens free of parasites, I conducted the first extensive examination of fungal parasitism of gardens of attine ants.

2.3 MATERIALS AND METHODS

2.3.1 Sampling of Attine Gardens

To assess the diversity and abundance of non-mutualistic filamentous fungi in attine ant gardens, I surveyed gardens tended by diverse attine lineages in central Panama from 1996 to 1998. Colonies were collected from the canal-region of central Panama, including Ancon Hill, Naos Island, Gamboa, Soberanía Park, Pipeline Road, and Fort Sherman Military Reservation. In total, the gardens of 201 colonies from 8 attine genera, representing the known phylogenetic diversity of the attines, were sampled for fungal parasites. Nests were

excavated as carefully as possible to ensure minimal disruption to the garden. Colonies were collected and subsequently maintained in sterile plastic containers with multi-chamber systems: an inner chamber for the garden, and a larger surrounding chamber as a foraging area. The ants were permitted to stabilize their garden for 3-5 days after collection before samples were taken.

Sampling of non-mutualistic fungi was conducted by placing small individual pieces of garden material (ca. 3mm³) on nutrient agar. In 1996, 20 pieces per garden were isolated, and 10 or 12 pieces per garden in 1997 and 1998. Pieces were selected from throughout the garden and placed on potato dextrose agar medium with antibacterial antibiotics (Penicillin-G and Streptomycin sulfate) under aseptic conditions. The pieces were monitored daily for the growth of non-mutualistic filamentous fungi; if arising from the inocula, these were then isolated into pure culture. Ants were not given forage material before isolations, minimizing the possibility of obtaining fungi present only as inoculum from recently added garden substrate.

Since the microbes isolated from attine gardens may represent inactive propagules (which may overgrow the mutualist only on nutrient agar), rather than *in situ* microbial activity, the long-term and consistent presence of non-mutualistic fungi in attine fungal gardens was examined. Representative species of different attines were maintained in the laboratory in the Botany Department of the University of Toronto to resample for persistent, non-mutualistic fungi including: 10 colonies each of *Apterostigma cf. pilosum*, *Cyphomyrmex longiscapus*, *Trachymyrmex cf. zeteki*, *Acromyrmex octospinosus*, *Atta colombica*, and *Atta cephalotes*. Colonies were maintained in the laboratory for a 10 month period. After initial sampling in the field, each colony was resampled every 4-8 weeks. Substrate material provided in the laboratory to ants as forage was confirmed to be free from the common non-mutualistic fungi obtained in the initial field sampling. Air-sampling of spores of these fungi

was conducted near ant colonies in Gamboa using a standard Biotest-Reuter centrifugal air-sampler (Biotest AG, Breieich, Germany). To reduce the possibility of infections spreading between colonies in the laboratory, instruments were sterilized before each new colony was fed or cleaned. Also, moat, mineral-oil, and physical barriers were used to prevent the spread of mites which potentially could vector fungal propagules between nests. Re-isolation of a fungus in the laboratory over an extended period of time was interpreted as evidence of chronic presence and growth within a garden.

2.3.2 Pathogenicity and Virulence of Garden Contaminants

To determine if *Escovopsis*, the most commonly isolated non-mutualistic fungus in the above mentioned sampling (see results), is a parasite of attine gardens, the virulence of fungi in this genus was investigated by (i) monitoring for colonies that have been over-grown by the fungus in the laboratory and field; (ii) excluding large garden fragments from tending ants to determine if, in the absence of the garden-tending, *Escovopsis* erupts and manages to over-grow the fragment; and (iii) directly inoculating colonies with *Escovopsis* and monitoring these intentionally infected gardens.

I infected fungal gardens with *Escovopsis* in the laboratory using young colonies of *A. colombica*, a common species of leaf-cutting ant in the canal region of Panama. Incipient colonies were collected in Gamboa, Republic of Panama, 6-8 weeks after the mating flight and were confirmed to be free from *Escovopsis*. Colonies were inoculated by spraying a suspension of fungal spores in sterile distilled water directly onto the garden with a mist-inoculator. Colonies (garden volumes of ca. 60-75 ml) were inoculated with *Escovopsis* at ca. 300-500,000 spores per colony. I sprayed another set of clean *A. colombica* gardens with ca. 400-600,000 spores of *Trichoderma*, an aggressive, necrotrophic fungal parasite that is common in tropical soil (Dix and Webster 1995). Since *Trichoderma* is an ubiquitous and aggressive fungus, out-competing *Escovopsis* in Petri dish bioassays (Currie unpublished

results), my spray treatment of *Trichoderma* controls for the effect of unnaturally high inoculation by aggressive fungi. Control colonies were sham treated with sterile water. Colonies were provided *ad libitum* access to forage substrata, and garden health was monitored twice daily. To complete Koch's postulates, the 'gold standard' in demonstrating pathogenicity (Agrios 1988), I attempted re-isolation of *Escovopsis* and *Trichoderma* after signs of garden deterioration were observed, or at the completion of the study period.

2.3.3 Transmission of *Escovopsis*

The transmission method of *Escovopsis*, horizontal (infection spread between established colonies) or vertical (infection passed from natal nests to newly established colonies), was examined using foundress queens and incipient nests of *A. colombica* in Gamboa, Panama in 1998. Foundress queens (gynes) were collected during the *A. colombica* mating flight that occurred on June 23, 1998. The fungal pellet was dissected from the infrabuccal pocket of 38 gynes and sampled for presence of *Escovopsis*. In addition, eight gynes, collected during the mating flight, were sampled after allowing them to establish gardens in the laboratory. To determine if *Escovopsis* contamination occurred *after* fungal pellets are spit-up under field conditions, but *before* foraging for vegetation begins by the first workers, 22 incipient colonies were marked and excavated during the claustral period (3-4 weeks after nest initiation, a time-span sufficient for a garden to become established, but during which lateral transfer cannot occur because queens plug the nest entrance and do not leave the garden chamber). Gardens of these claustral nests were sampled for *Escovopsis*. Incipient colonies (n=45) that had begun foraging, identifiable by the emergence of a small turret surrounding the nest entrance, were also excavated and their gardens sampled. Finally, one- or two-year-old colonies were sampled for *Escovopsis* to establish a base-line infection rate for young *A. colombica* colonies in this population.

2.3.4 Intergeneric Comparison

My extensive and representative sampling of alien fungi from fungal gardens of the ants, in conjunction with the recently published phylogeny of the Attini (Schultz and Meier 1995, Wetterer 1998), permits an examination of parasitism throughout the generic diversity of this tribe. This analysis was based on sampling from 182 attine colonies, and a total of 2,480 pieces of fungal garden. Some of the ant species and their corresponding genera sampled for non-mutualistic fungi were of low abundance, so insufficient sample sizes were obtained to warrant inclusion (i.e. *Myrmicocrypta* sp., n=5; and *Sericomyrmex* sp., n=6), while others were extremely difficult to collect without significant disturbance (i.e. *Mycocepurus* spp. n=8). Thus the comparative examination was concentrated on collections from five attine genera: *Apterostigma* (n=30), *Cyphomyrmex* (n=35), *Trachymyrmex* (n=39), *Acromyrmex* (n=29), and *Atta* (n=49). Several species were studied for each genus to ensure more representative sampling, as follows: *Apt. auriculatum*, *Apt. cf. pilosum* sp. 1, *Apt. cf. pilosum* sp. 2, *C. longiscapus*, *C. costatus*, *T. cf. zeteki*, *T. cornetzi*, *T. bugnioni*, *Ac. octospinosus*, *Ac. echinator* (Schultz *et al.* 1998), *Atta colombica*, and *Atta cephalotes*. Both the proportion of pieces contaminated with non-mutualistic fungi (a measure of the prevalence of potential parasites) and the prevalence of *Escovopsis* as a portion of total garden contamination were compared across the 5 genera.

2.4 RESULTS

2.4.1 Sampling of Attine Gardens

Attine gardens are occupied by numerous filamentous fungi aside from the mutualist. Of the 2,480 individual pieces of attine fungal garden sampled over the three years of this study, 39.7% of the pieces yielded non-mutualistic fungi, totaling 984 fungal cultures. Specifically, contamination ranged from a high of over 54.8% of pieces from gardens of *Apterostigma* spp. colonies to a low of 27% from *Atta* spp. gardens (Fig. 2.1a). The fungal genus

Escovopsis was second only to the mutualist in abundance. *Escovopsis* was the major taxon of non-mutualistic fungi isolated within infected colonies, ranging from 28.1% to 77.5% of contaminants, depending upon the ant genus (Fig. 2.1b). This genus accounted for 256 of the 984 contaminants obtained from these samples (26.0%), was isolated from all 8 attine genera examined, and infected, a minimum of, 33.3% to 51.5% of the ant colonies sampled (Table 2.1). Using morphological characters, these 256 isolates of *Escovopsis* appear to comprise at least 8 distinct undescribed species (Currie unpublished results), a technical account of which will be published elsewhere.

Colonies of *Apt. cf. pilosum* sp. 1 (n=3), *C. longiscapus* (n=4), *T. cf. zeteki* (n=5), *Ac. octospinosus* (n=3), *Atta colombica* (n=4), and *Atta cephalotes* (n=3) naturally colonized by *Escovopsis* showed persistent infection after maintenance in the laboratory for 6, 3, 8, 10, 10, and 10 months, respectively. *Escovopsis* was not isolated in air-samples near nests in the field and laboratory, nor from forage material provided to the ants, so the observation of a long-term sustained presence indicates proliferation (growth) of this fungus within gardens.

2.4.2 Pathogenicity and Virulence of Garden Contaminants

Escovopsis can be a virulent pathogen of attine fungal gardens. The fungus has been observed to over-grow gardens, even in the presence of the ants, both in the field (see Fig. 2.2) and in the laboratory (also see Möller 1893, Weber 1966). Maintaining garden material in the absence of ants (from infected colonies) almost invariably leads to rapid (1-2 days) *Escovopsis* over-growth. I observed this in all *Escovopsis*-infected garden material maintained in isolation from tending ants of the three higher attines, *T. cf. zeteki* (n=10), *Ac. octospinosus* (n=6), and *Atta colombica* (n=10). Contrary to anecdotal suggestions in the literature that removal of tending ants leads to rapid over-growth of the garden by multiple different contaminants (cf. Weber 1966, Fisher *et al.* 1996), gardens with endemic *Escovopsis* were only over-grown by this fungus. In addition, colonies judged free of

Escovopsis were over-grown by other fungi only 7-14 days following the removal of the tending ants. After intentionally infecting 16 *A. colombica* gardens with spores of *Escovopsis*, gardens of six colonies collapsed and were lost within 72 hours (see Fig. 2.2 for a collapsed, *Escovopsis* infected *Trachymyrmex* garden), and three of the remaining 10 colonies lost their gardens within 9, 13, and 17 days, respectively. None of the colonies treated with *Trichoderma* or sterile water showed signs of garden loss or even noticeable signs of garden stress. *Escovopsis* was re-isolated from experimentally infected colonies; however, attempts to re-isolate *Trichoderma* were unsuccessful.

2.4.3 Transmission of *Escovopsis*

Escovopsis is not vertically transmitted in *A. colombica*. New queens were found to carry axenic inocula of the mutualist during mating flights, all incipient gardens were free of *Escovopsis* during the claustral phase of colony foundation, and only a small percentage of nests (6.6%) contained *Escovopsis* shortly after the commencement of foraging (Table 2.2). In contrast, *Escovopsis* was isolated from almost 60% of the 1-2 year old colonies of *A. colombica* in the same population. Thus, transmission of *Escovopsis* appears to be exclusively horizontal.

2.4.4 Intergeneric Comparison

Two distinct evolutionary trends emerged from my examination of non-mutualistic fungi in attine gardens. First, total prevalence of non-mutualistic fungi decreases across the phylogeny from the basal genera to the more derived genera (Fig. 2.1a). Secondly, there is a greater prevalence of *Escovopsis* within colonies of the more derived attines, even though overall general contamination (with microfungi of any kind) decreases in these species in comparison to the more basal attines (Fig. 2.1b).

2.5 DISCUSSION

The mutualism between fungus-growing ants and their fungi is parasitized by a fungus in the hyphomycete genus *Escovopsis*. The parasitic nature of this fungus is revealed by two findings: first, *Escovopsis* is a common, prolific, and persistent microbe in the gardens of attine ants; second, inoculations of healthy garden with *Escovopsis* spores revealed the pathogenicity of *Escovopsis*. Specifically, experimentally infected gardens showed high morbidity and mortality, establishing that this pathogen has the potential to be a virulent parasite of the mutualism. As speculated by Seifert *et al.* (1995), the genus *Escovopsis* is specialized on fungal gardens of attine ants, as it has not been isolated from any other habitats (see Seifert *et al.* 1995, Muchovej and Della Lucia 1990), and it was not obtained in air-samples or samples of forage material in this study. *Escovopsis* is consistently present in gardens of fungus-growing ants that span the breadth of the attine phylogeny, having been isolated from all 8 genera examined in this study. In addition, this fungus occurs throughout the range of the attines, having been isolated from attine ant gardens from Brazil, Ecuador, Guyana, Panama, Texas and Trinidad (Seifert *et al.* 1995, Muchovej and Della Lucia 1990, Currie unpublished results). Although only two species of *Escovopsis* have been previously described (each was isolated on one occasion from a single attine garden; see Muchovej and Della Lucia 1990), the specific habitat of this fungus and the limited scope of previous mycological studies of attine gardens has apparently resulted in an underestimation of the species diversity of the genus.

The exact mechanisms of pathogenicity by *Escovopsis* remain unclear. Preliminary evidence suggests that *Escovopsis* may be a mycoparasite, obtaining nutrients only from the mycelium of the ant-cultivated fungus, but not from the vegetative material of the garden. In both gardens with workers removed and in bioassays challenging *Escovopsis* against the fungal mutualist, *Escovopsis* was observed to overgrow the fungal mutualist quickly and

completely, leaving no apparent microscopic evidence of the mutualist fungus. This suggests that *Escovopsis* excretes lytic enzymes and/or toxins, a common trait among necrotrophic mycoparasitic fungi (Dix and Webster 1995), thus supporting a mycoparasitic interpretation. Alternatively, it is possible that *Escovopsis* is a highly evolved “weed” that parasitizes this mutualism by competitively inhibiting the fungal mutualist after colonizing the vegetative garden substrate. Further work should attempt to differentiate between the “mycoparasitic” and the “weed-parasitic” mechanisms of *Escovopsis* pathogenicity.

Microbes are ubiquitous upon and within the vegetative material added to the fungal garden of attine ants. These fungi and bacteria, including competitive saprotrophs and aggressive necrotrophs, are apparently suppressed by the ants through their continuous garden-tending activities (Schildknecht and Koob 1970, Martin 1987, Bass and Cherrett 1994) and the antimicrobial defenses of the mutualist fungi (Hervey and Nair 1979, Wang *et al.* 1999). Thus these “generalist” microbes, which by definition have not evolved specializations to circumvent the generalized behavioral and chemical defenses of the ants, do not persist, proliferate, or indeed impact the garden. They are functionally equivalent to un-germinated seeds in human agricultural soils, and as such, their presence as passive occupants does not refute the near axenic monoculture hypothesis. Unlike these “generalist” microbes, however, *Escovopsis* is a specialist that apparently has evolved specializations to circumvent the defenses of the ants. It readily proliferates and persists in the colonies, with a high potential for devastating attine ant gardens, despite careful tending by the ants. Therefore, the documented chronic presence and growth of *Escovopsis* shows, for the first time, that fungal gardens of attine ants are not maintained in near axenic “monocultures”.

In addition to the identification of a specialized and virulent parasite of the garden, my findings suggest an evolutionary cost of long-term clonality of cultivars in terms of increased severity of infections by specialist parasites. In the only well-studied analogous system, human agriculture, large and genetically homogeneous cultivars are extremely susceptible to devastating epidemics, which at times have shaped human history and culture (e.g. the Irish potato famine; Lucas 1980, Barrett 1981). Such susceptibility to pathogens is thought to be a result of the cultivars' limited genetic diversity, which facilitates rapid specialization, attack, and spread of diseases. Such pathogen adaptation can occur extremely quickly; for example, blue mold has been observed to devastate homogeneous crops of newly introduced 'resistant' tobacco cultivars in less than three years (Lucas 1980).

An evolutionary cost of long-term cultivar clonality among fungus-growing ants is supported by my finding of an increased prevalence of *Escovopsis* within gardens of the more derived genera, possibly because of evolution toward increased virulence by *Escovopsis*. The increased prevalence of *Escovopsis* within the more derived attine genera suggests that the long clonal history of these fungal cultivars, perhaps as long as 23 million years (Chapela *et al.* 1994), makes them more susceptible to losing the "arms race" with parasites (Jaenike 1978, Hamilton 1980). By contrast, lower attines routinely acquire new fungal cultivars from free-living, sexual populations (Mueller *et al.* 1998), leading to greater genetic diversity in the fungal mutualist population. This may account for the apparent lower susceptibility to parasitism of the less derived attine lineages.

Biologists have long been fascinated not only by the farming life-history of the attines and the conspicuous foraging trails of the leaf-cutting ants, but also by the interesting evolutionary trends represented by extant assemblages of ants. There are distinct phylogenetic trends in the attines for increasing worker size, worker polymorphism, and

colony size (Hölldobler and Wilson 1990). This has led to the hypothesis that there has been an evolutionary improvement in fungal cultivation across the attine ants (Hölldobler and Wilson 1990, Wheeler 1910). My finding of decreasing abundance of non-mutualistic “generalist” fungi across the phylogeny of the fungus-growing ants is the first empirical evidence supporting the hypothesized improved fungal cultivation across the attine phylogeny.

Since leaf-cutting ants and their fungi are obligately mutually dependent, the success of this highly evolved mutualism depends upon the maintenance of stable cultures. This predicts that successful maintenance of gardens in fungus-growing ants involves a continual struggle to suppress the specialized fungal parasites *Escovopsis*. The mechanism of this suppression has recently been identified in the form of a third mutualist associated with both higher and lower attine ants, an actinomycete which produces antibiotics that specifically target and inhibit the growth of *Escovopsis* (section 4.0 or Currie *et al.* 1999). Thus, it appears that, through chemical intervention, attine ants are able to cultivate successfully genetically homogeneous fungal gardens. Further studies on the interaction of garden parasites should provide additional insight into the evolution of this fascinating mutualism. Moreover, the discovery of a highly virulent parasite of the ant gardens of the attines may open new avenues of biological control of this devastating agricultural pest in the New World tropics.

Table 2.1 Proportion of attine colonies parasitized by *Escovopsis* from sampling in Panama, 1996 to 1998

Attine genus	Number sampled	Gardens infected with <i>Escovopsis</i> (%)
Apterostigma	30	33.0%
Cyphomyrmex	35	45.7%
Trachymyrmex	39	51.1%
Acromyrmex	29	51.4%
Atta	49	42.9%

Table 2.2. Isolation of *Escovopsis* spp. from different life history stages of *A. colombica*

<i>A. colombica</i> life history stage	Number sampled	Gardens infected with <i>Escovopsis</i> (%)
Foundress queens: from infrabuccal pocket	38	0%
Foundress queens: gardens established in the laboratory	8	0%
Incipient colonies: before commencement of foraging	22	0%
Incipient colonies: after commencement of foraging	45	6.6%
1 to 2 year old Colonies	24	58.3%

Figure 2.1 Proportion of garden pieces isolated that contained non-mutualistic filamentous fungi and the proportion of non-mutualistic fungi belonging to the genus *Escovopsis*. The attine genera are positioned from the most basal genus to the most derived genus (see Schultz and Meier 1995, Wetterer *et al.* 1998). (Error bars represent standard errors, n=number of colonies sampled, p=total number of pieces sampled).

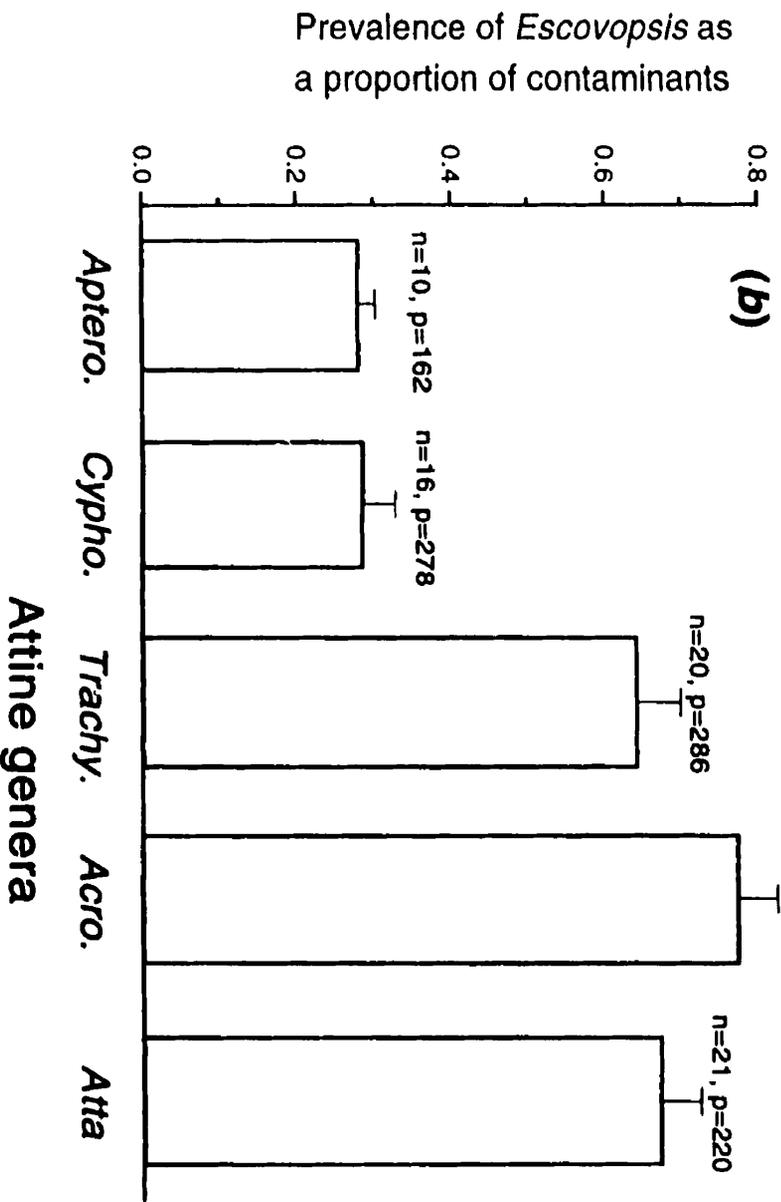
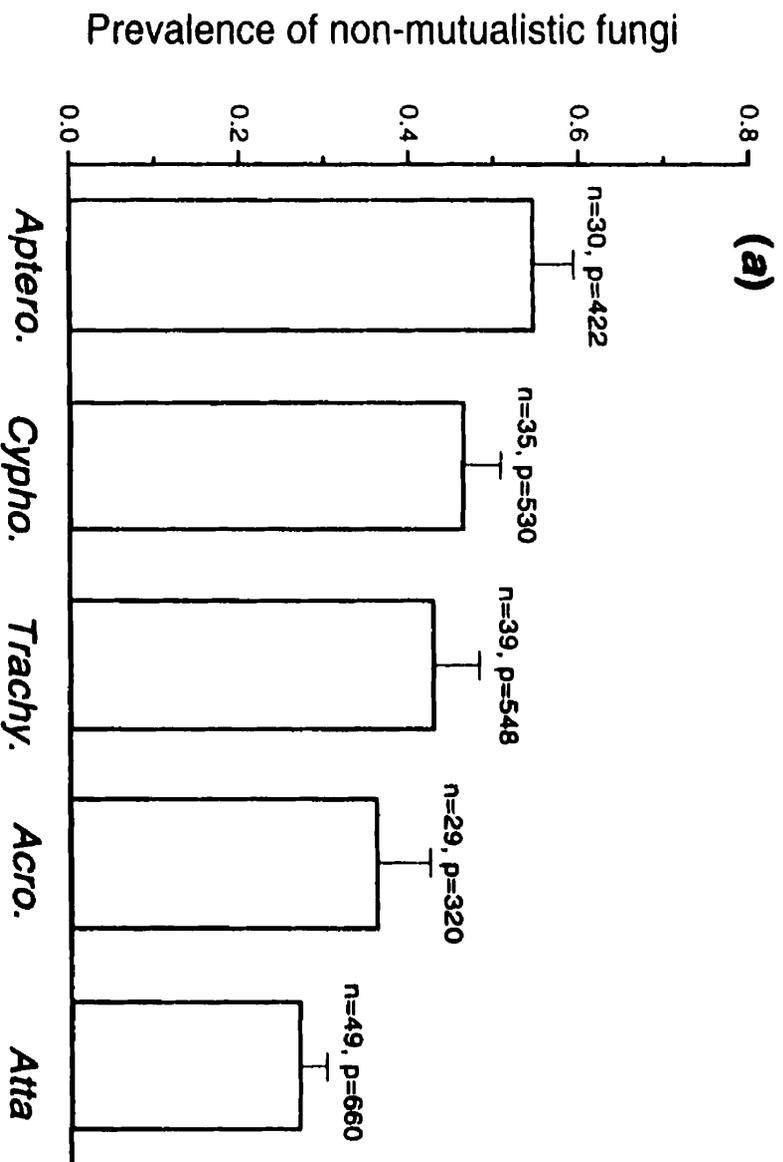


Figure 2.2 (A) A healthy *Trachymyrmex* sp. garden and (B) a completely devastated *Trachymyrmex* sp. garden overgrown by the parasite *Escovopsis*.



3.0 PREVALENCE AND IMPACT OF A VIRULENT PARASITE ON A TRIPARTITE MUTUALISM

3.1 ABSTRACT

The prevalence and impact of a specialized microfungus parasite (*Escovopsis*) that infects the fungal gardens of leaf-cutting ants was examined in the laboratory and the canal region of Panama. *Escovopsis* is a common parasite of leaf-cutting ant colonies, and it is apparently more frequently present in *Acromyrmex* spp. gardens than in gardens of the more phylogenetically derived genus *Atta* spp. In addition, colonies of *Atta cephalotes* in Gamboa had a much higher infection rate than their conspecifics in Fort Sherman Military Reservation, suggesting that *Escovopsis* is geographically aggregated. Older colonies of *Atta* spp. appeared to be less frequently infected with the parasite; however, other results of this study challenge the traditional method of aging colonies. In this study, the parasite *Escovopsis* had a major impact on the success of this mutualism. Infected colonies had a significantly lower rate of fungus-garden accumulation and produced substantially fewer workers. In addition, the extent of the reduction in colony growth rate depended on the isolate, with one strain having a significantly larger impact than the other two. This indicates that *Escovopsis* has different levels of virulence, even within the same populations as all three cultures were obtained from this population. *Escovopsis* is also spatially aggregated within fungal-gardens, with the younger regions having significantly lower rates of infection as compared to the older regions.

3.2 INTRODUCTION

The importance of parasites, especially disease producing organisms, in shaping all levels of biological organization is widely recognized (Price 1986, Anderson and May 1991, Herre 1995). Parasites even influence other interspecific interactions, such as competition and predation (Freeland 1983, Price 1986, Schall 1992, Yan *et al.* 1998). Within mutualistic association, most of the research on parasites has focused on 'cheaters', taxa that are closely related to one of the mutualists but do not co-operate, obtaining a reward without providing a benefit in return (Boucher *et al.* 1982, Mainero and Martinez de Rio 1985). The interest in 'cheaters' within mutualisms is at least partially based on the stability of co-operation being a challenge to evolutionary theory (e.g., Morris 1996, Pellmyr *et al.* 1996, Addicott 1996, Bao and Addicott 1998). However, few studies have examined the importance of parasites that are unrelated taxa that have evolved to exploit mutualisms, rather than 'cheaters'. In this study, I examine the impact of the microfungus parasite *Escovopsis* on the tripartite mutualism occurring among leaf-cutting ants (Formicidae: Attini), their fungal cultivars, and symbiotic filamentous bacteria (actinomycetes).

This tripartite mutualism likely originated around 50-65 million years ago (Wilson 1971, Mueller *et al.* submitted). The fungi, mostly belonging to the family Lepiotaceae (Basidiomycota: Agaricales) (Chapela *et al.* 1994, Mueller *et al.* 1998), serve as the primary food source for the ants. The filamentous bacteria help protect the fungal gardens from specialized parasites, and may promote the growth of the fungus in some taxa (section 4.0 or Currie *et al.* 1999a, section 5.0). The ants forage for new substrate to manure the garden (including plants, insect frass, or seeds), engage in elaborate garden-tending behaviors, and disperse both the fungus and bacteria to new colonies through vectoring by the foundress queens (von Ihering 1898, Huber 1905, section 4.0 or Currie *et al.* 1999a).

The ant tribe Attini includes ca. 210 species and 12 genera (Weber 1966, Schultz and Meier 1995), but only the two most derived genera (*Acromyrmex* and *Atta*) use fresh vegetative material for manuring their gardens. The latter genera are commonly referred to as leaf-cutting ants. Species in the leaf-cutting ant genus *Acromyrmex* have mature colonies with populations of thousands of workers, typically with one large fungal garden (Weber 1966, Wetterer 1999). Leaf-cutting ants in the genus *Atta* have the most complex fungicultural systems, which are so successful that mature colonies in the genus *Atta* can support millions of workers and hundreds of fungal gardens (Weber 1966). The combined foraging activity of the leaf-cutters causes more defoliation than almost any other herbivore in the neotropics (Hölldobler & Wilson 1990).

The process of colony reproduction begins with synchronously timed production of reproductive alates (both queens and males), which mate during nuptial flights that typically occur on one or two days at the beginning of the rainy season. Foundress queens of the leaf-cutting ants dig subterranean chambers and begin the cultivation of their new gardens from a fungal pellet from their natal nests. This fungal inoculum is stored in the queens' infrabuccal pockets. Initially these incipient colonies remain sealed within the claustral chamber, where the queens use fecal material to maintain the growth of the garden. It is not until the first brood is reared that the new workers begin foraging for leaf material outside the chamber. Colonies continue to accumulate larger biomass of both garden, through the continuous addition of leaf-material and growth of the fungus, and ants. The life-cycle of the colony is only completed once sufficient colony size is reached to support the production of new reproductives (alates). Reaching this threshold typically requires hundreds of thousands of workers and dozens of individual fungal gardens (Weber 1966, 1972). Leaf-cutting ant colonies can live for 10 years or more after initial nest founding (Weber 1966).

Reproductively mature colonies of the leaf-cutting ants can produce thousands of alates annually, yet only an extremely small proportion of queens successfully establish new mature colonies (Weber 1966, 1972). The etiology of this extremely high rate of alate and incipient colony mortality has only partially been established. Predation of gynes (female reproductives) by birds and bats during nuptial flights is extremely high (Autuori 1950, Water *et al.* 1962, Mariconi 1970, Fowler *et al.* 1986). The gynes surviving to begin digging chambers in the soil are also subject to heavy predation by birds, mammals, and ants (Mariconi and Zamith 1963, Maroconi 1970, Weber 1972). In addition, unsuccessful establishment of fungal gardens by foundress queens may be a frequent event (Autuori 1941). Once the garden is established, the incipient colonies are still vulnerable to attack by army-ants and already established leaf-cutting ant colonies (Cherrett 1968, Rockwood 1973, Fowler 1982, Fowler *et al.* 1984). Leaf-cutting ant colonies need to grow quickly in order to fend off attacks by predators, including other ant colonies, to reach reproductive maturity, and potentially to successfully compete for leaf material with other *Atta* colonies in the population (Weber 1972). This strong selection for fast colony growth has apparently led to the evolution of exponential growth rates in the leaf-cutting ant colonies, especially in the genus *Atta*, which can reach reproductive maturity with colony sizes in the millions of workers ca. 3 years after initial nest founding (Weber 1966, 1972, Hölldobler and Wilson 1990).

New research has identified that fungal gardens of leaf-cutting ants are host to a potentially virulent pathogen in the microfungus genus *Escovopsis* (section 2.0 or Currie *et al.* 1999b). This pathogen is common among colonies, can be prolific within colonies, leads to colony death under some conditions, and is able to persist within the fungal garden for extended periods of time (section 2.0 Currie *et al.* 1999b). Although it is clear that *Escovopsis* has an impact on the survivorship of leaf-cutting ants, the effects of this parasite on the success of this tripartite mutualism have not been investigated. Therefore, I examined the prevalence

and impact of *Escovopsis* on the leaf-cutting ants in central Panama. More specifically, I examined the following questions: i) is the prevalence of *Escovopsis* different among sympatric species of leaf-cutting ants; ii) is the prevalence of *Escovopsis* spatially aggregated geographically across the isthmus of Panama; iii) do different age colonies have different rates of infection, iv) do persistent infections of *Escovopsis* reduce the growth rate of colonies, and v) are infections within colonies spatially aggregated.

3.3 MATERIALS AND METHODS

3.3.1 Prevalence of *Escovopsis*

First, I examined the prevalence of *Escovopsis* within the fungal gardens of leaf-cutting ants in Gamboa, Panama. Gamboa is located in the canal-region of the Panamanian isthmus, approximately 30 km north of Panama City. This site is composed of secondary tropical moist forest. Sampling was conducted on four common species of leaf-cutting ants in Gamboa: *Acromyrmex octospinosus* ($n = 19$), *Acro. echinator* ($n = 8$), *Atta colombica* ($n = 33$), and *A. cephalotes* ($n = 14$) (Weber 1969, Schultz *et al.* 1998).

The presence/absence of *Escovopsis* within colonies was determined through the collection of an equivalent amount of garden material from each colony (ca. volume of 200-250 ml). Since incipient colonies typically are not infected with the parasite (section, 2.0 or Currie *et al.* 1999b), they were not included in this study. Garden material was collected by carefully excavating the soil and extracting a single garden chamber, including all the workers tending it. Care was taken to cause minimal disruption to the fungal garden. These sub-colonies were maintained in the laboratory for 3-5 days prior to sampling for *Escovopsis*. Sampling was conducted by placing small individual pieces of garden material (ca. 3mm²) on nutrient agar. Initially, 10 pieces per garden were isolated. If no *Escovopsis* was obtained, absence of infection was confirmed with an additional sampling of 10 pieces. Pieces were isolated from throughout the garden and monitored daily, as outlined in Currie *et al.* (1999b). All

isolations were done on potato dextrose agar (PDA) medium (Difco, Detroit, Michigan) with antibacterial antibiotics (ca. 50 mg/L of Penicillin-G and Streptomycin sulfate) under aseptic conditions.

To examine the possibility that the parasite *Escovopsis* is geographically aggregated within the leaf-cutting ant colonies across the Panamanian isthmus, I conducted additional sampling of colonies in Fort Sherman Military Reservation (FSMR) and on Barro Colorado Island (BCI). No colonies of *Acromyrmex* were found at either site, and therefore no representatives of this genus were included in this part of the study. At FSMR, 30 leaf-cutting ant colonies (*A. cephalotes* $n = 11$ and *A. colombica* $n = 19$) were sampled for *Escovopsis* using the same methods outlined above for colonies located in Gamboa. BCI is a Republic of Panama Nature Monument, so destructive sampling is prohibited. However, *A. colombica* is an abundant leaf-cutting ant on BCI, and is unique among *Atta* spp. in Panama in that the workers carry the refuse (dump) material outside of the colony, placing it on the forest floor. Sampling for *Escovopsis* in colonies of *A. colombica* was therefore possible on BCI by isolating from refuse material (composed of old garden material) as it was carried to the dump. In fact, this may be a more reliable method for assessing infection of colonies since workers carry refuse material from throughout the colony, allowing a broader sampling of the whole biomass of garden (see Bot *et al.* submitted). Sampling was done by aseptically removing the small pieces of refuse material (ca. 4-5 mm³) from workers immediately after they emerged from the colonies. In total, 24 pieces of dump material were sampled from each colony ($n = 53$ colonies) and placed on PDA medium. As with garden material, refuse pieces were monitored daily in Petri plates for the growth of non-mutualistic filamentous fungi; if arising from the inocula, these were then isolated into pure culture and confirmed to be *Escovopsis*.

3.3.2 Age of colony and infections of *Escovopsis*

The relationship between age of colony and presence of *Escovopsis* was examined in 1997 in Gamboa and FSMR. Since it is difficult to age colonies of *Acromyrmex*, only *Atta colombica* and *A. cephalotes* were included in this part of the study. In total, the age and presence of *Escovopsis* was estimated for 42 colonies of *Atta* spp. from FSMR ($n = 30$) and Gamboa ($n = 12$). Estimate of colony age was based on the number of colony openings, the surface area on the forest floor occupied by the colony, and/or total size of garden (in the case of distinguishing incipient from 1-2 year old colonies). A logistic growth rate in *Atta* spp. colonies has been established suggesting that all of the above characteristics increase exponentially with the age of the colony (Bitancourt 1941). In addition, new colonies are only established following regionally synchronized nuptial flights, so all newly founded colonies within the same season are approximately the same number of days old.

3.3.3 Impact of *Escovopsis* on colony growth rate

To determine if the parasite *Escovopsis* has an impact on the growth rate of leaf-cutting ant colonies, incipient colonies of *A. colombica* were collected 8-10 weeks after their mating flight in Gamboa, Panama. Incipient colonies were collected because *Escovopsis* is a horizontally transmitted parasite (section 2.0 or Currie *et al.* 1999b), thus young colonies are typically free of infection. Collected colonies were confirmed to be free of *Escovopsis* infection and subsequently maintained in sterile plastic containers. An inner chamber was used to house the garden, and an outer chamber was provided for foraging and dumping refuse material. Colonies were maintained in the laboratory for two weeks prior to treatment, allowing them to stabilize. It is difficult to determine the initial garden mass or the number of workers within colonies without causing a major disturbance and subsequent loss of a significant amount of garden material. Therefore, a comparison was made of the final garden

mass and numbers of ants rather than of the growth rates. Since all incipient colonies are approximately the same size and treatments were randomly allocated, differences in initial garden mass would not affect the results obtained in this study.

Colonies were randomly assigned to one of four treatments. Three groups of colonies were infected with the garden parasite, each group being treated with a different strain of *Escovopsis*. Prior to the experiment, the three strains of *Escovopsis* were isolated from 3 different *A. colombica* colonies from the same population of leaf-cutting ants as those used in this experiment. Colonies were infected with *Escovopsis* by using a mist inoculator with a fungal spores suspension in sterile distilled water. Each colony was sprayed with ca. 40-60,000 spores. The fourth group consisted of control colonies, which were sprayed with sterile distilled water.

Significant precautions were taken to prevent the cross-contamination of *Escovopsis* between colonies. *Escovopsis* is a wet-spored fungus, so air-borne contamination is extremely unlikely; however, vectoring by either mites moving between colonies or human contact during the maintenance of the colonies is possible. To prevent the movement of mites between colonies, uninfected and infected colonies were maintained on different, spatially separated tables. In addition, the outer surfaces of each colony's outer chamber were covered in fluon (Northern Products, Inc., Rhode Island), and a small film of heavy mineral oil was applied to the upper edge of the inner surface of each colony. To prevent human vectoring of *Escovopsis*, uninfected colonies were always handled first. In addition, sterile techniques (i.e. washing hands, sterilizing forceps, etc) were employed rigorously during maintenance of colonies. Colonies were provided *ad libitum* access to fresh vegetation for foraging, and

water was added to a small dish in the outer chamber once per week. The colonies were maintained in an open-air laboratory in Panama.

Colonies were maintained under the conditions outlined above for 7 weeks. To determine the impact of *Escovopsis* infection on the growth rate of leaf-cutting ant colonies, the garden mass and number of workers, larvae, and pupae was determined at the end of the experimental period. The 'wet-weight' of the garden was measured, which is strongly correlated to 'dry-weight', and allows post-weighing sampling of the garden material for *Escovopsis* (see below). The presence or absence of *Escovopsis* was confirmed by isolating 24 garden pieces on PDA (as described above) in infected and control colonies, respectively. The effect of treatment on these variables was compared statistically with an ANOVA using JMP (version 3.2.2, SAS Institute 1997). The effect of different isolates of *Escovopsis* was also compared using an ANOVA with isolate nested in treatment in the model. Normality of the data was confirmed using the Shapiro-Wilk W test. The variable 'larvae' was non-normal and therefore was transformed using the natural log.

3.3.4 Location of *Escovopsis* within gardens

To examine if infection of *Escovopsis* is spatially aggregated within gardens, the prevalence of the parasite in different locations within the infected colonies (in the above mentioned experiment) was examined. At the end of the 7 week period, 24 pieces of garden were isolated from each of the infected colonies, with 7 pieces isolated from each of the top, middle, and bottom of gardens. The prevalence of infection across these garden areas was statistically compared by means comparisons using the Tukey-Kramer HSD test with JPM (version 3.2.2, SAS Institute 1997).

3.4 RESULTS

3.4.1 Prevalence of *Escovopsis*

The proportion of colonies infected with *Escovopsis* was very similar between the congeneric leaf-cutting ant species occurring in Gamboa (Table 3.1.). However, in Gamboa, *Escovopsis* was isolated more from colonies of *Acromyrmex* than *Atta*, with at least 70.4% and 48.9% of the colonies infected, respectively (Table 3.1).

The frequency of *Escovopsis* isolation in colonies of *Atta* differed between the three locations, suggesting that the parasite is more prevalent in some locations for some species. For example, only 18.2% of *A. cephalotes* colonies were infected in FSMR as compared to 50.0% of colonies of this species in Gamboa (Table 3.1). In addition, a higher proportion of colonies of *A. colombica* on BCI were infected than in Gamboa and FSMR (Table 3.1). However, this could be a sampling artifact, as isolating from the dump is not equivalent to isolating directly from the garden.

3.4.2 Age of colony and presence of *Escovopsis*

My sampling of *A. cephalotes* and *A. colombica* colonies of different ages suggests that *Escovopsis* is less prevalent in older colonies (Figure 3.1). More than 45% of 1-2 year old *Atta* spp. colonies were infected with this parasite as compared to only ca. 20% of colonies that are 5 years old or older.

3.4.3 Impact of *Escovopsis* on colony growth rate

The presence of *Escovopsis* within the fungal gardens of leaf-cutting ants has a negative effect on the growth and health of colonies. In this study, colonies of *A. colombica* infected with *Escovopsis* had significantly smaller final garden mass as compared to uninfected colonies (Figure 3.2a; $F=19.63$, $df=1, 14$, $P<0.0006$). The garden size of uninfected colonies was on average twice as large as infected colonies.

Infections of *Escovopsis* within *A. colombica* gardens also had a significant impact on the production of new workers within colonies. Infected colonies had significantly fewer larvae, pupae, and adult ants than the uninfected control colonies at the end of the experimental period (Figure 3.2b,c,d; $F=21.92$, $df=1$, 14 $P<0.0002$; $F=24.52$, $df=1$, 14 , $P<0.0004$; and $F=27.65$, $df=1$, 14 , $P<0.0001$, respectively).

The impact of *Escovopsis* infection on the health of the colony was also affected by the isolates of parasite used in this study. No significant difference in effect on garden mass was observed between the three isolates used in the experiment (isolate nested within treatment: $F=1.19$, $df=2$, 14 , $P<0.3350$). However, a significant effect of *Escovopsis* isolate was obtained for the final number of larvae and adult ants within colonies (isolate nested within treatment: $F=10.21$, $df=2$, 14 , $P<0.0371$ and $F=4.21$, $df=2$, 14 , $P<0.0018$, respectively). A marginal effect of *Escovopsis* isolate on the number of pupae was observed in this study (isolate nested within treatment $F=3.15$, $df=2$, 14 , $P<0.0743$).

3.4.4 Location of *Escovopsis* within gardens

The presence of *Escovopsis* within infected colonies is spatially aggregated. *Escovopsis* was most prevalent in the bottom and middle portions of the garden and least prevalent in the top, or youngest area, of the garden (Figure 3.3, $P < 0.05$ Tukey-Kramer HSD). These results suggest that infections are maintained in the lower or older regions, spreading upward as new leaf material is added. No significant difference between the middle and bottom of garden in prevalence of *Escovopsis* was observed.

3.5 DISCUSSION

Escovopsis has a significant impact on the success of the tripartite mutualism among leaf-cutting ants, their fungal cultivars, and filamentous bacteria. Infected colonies can be

completely over-whelmed by this fungus (Möller 1893, section 2.0 or Currie *et al.* 1999b), and, as seen in this study, persistent infections of the garden have a dramatic negative impact on the growth rate of colonies, both in terms of fungus-garden mass and workers.

My findings of a significant reduction in fungus-garden growth rate in *Escovopsis* infected colonies suggests this parasite has a major negative effect on the fitness of leaf-cutting ant colonies. Colonies must accumulate a vast amount of fungus-garden (i.e., hundreds of individual garden chambers) to support the production of reproductive alates (Weber 1966, Weber 1972), therefore any significant decrease in the growth rate will result in a significant delay in reproduction. *Escovopsis* may even prevent some colonies from ever reaching reproductive maturity by severely limiting the accumulation of fungus garden. In addition, the decrease in growth rate of the garden could have serious indirect effects on the success of this mutualism. For example, small colonies are more susceptible to being killed by army ants or larger leaf-cutting ant colonies, and are likely competitively inferior.

My finding of significantly fewer brood in infected colonies compared to uninfected colonies at the end of the experiment is likely due to a decrease rate of egg laying by the queen. However, it is also possible that eggs, and even larvae, may have been consumed by workers, as occurs in incipient colonies (Weber 1966, Weber 1972). Under the apparent stressful conditions of having a highly virulent parasite within the garden, this behaviour decreases the demands on the fungal mutualist by reducing the amount of brood that must be supported, and additionally provides some nutrients back to the colony. Infected colonies having fewer workers at the end of the experimental period are likely a result of fewer individuals being

produced because of the stressed fungal garden. However, this may not be a sufficient explanation to account for the major differences in workers observed as it may take as long as seven weeks, the duration of this experiment, for eggs to completely develop into adult ants (Weber 1966, Weber 1972). This suggests that the stress on the colony resulted in higher levels of mortality in adult ants. Unfortunately, mortality rates in workers was not examined in this study, so the impact of infection on workers survivorship cannot be examined.

At first glance, finding a lower frequency of *Escovopsis* in older leaf-cutting ant colonies suggests that infected colonies are more likely to die off before reaching a reproductively mature age as compared to uninfected colonies in the same age cohort. However, this incorrectly assumes colonies only become infected at an early age, not continuously, as would be expected with a horizontally transmitted parasite such as *Escovopsis*. Although it is possible that older colonies are more resistant to becoming infected by the parasite, a more likely explanation is that only colonies with an inherent resistance to *Escovopsis* reach these large sizes. My aging of colonies was based on an assumption of a consistent logistic growth rate in colonies, however, I show in this study that colonies infected with the parasite *Escovopsis* have a dramatically slower rate of colony growth (i.e., it is not valid to age colonies based on size). This suggests that the colonies that were assumed to be small because of age were potentially small because of a significantly reduce growth rate due to the persistent infection of *Escovopsis*.

The impact a parasite has on its host is referred to as its virulence. My results indicate that the parasite *Escovopsis* has different levels of virulence, as all three isolates of *Escovopsis* were obtained from the same population from which the experimental colonies were collected, but there was a significant difference in their impact on the number of adults and larvae in the colonies. The different strains of *Escovopsis* within the same population must compete between *Escovopsis* strains, between colonies and within colonies. Large *A. colombica* colonies may be composed of up to hundreds of individual chambers of fungal gardens, and since *Escovopsis* is horizontally transmitted (section 2.0 or Currie *et al.* 1999b), it is likely that within these large colonies different strains of pathogen are present in different garden chambers, or even within the same garden. Studies on the evolution of virulence in *Escovopsis* could provide theoretical insight into the evolution of parasites and pathogens in general.

Workers continuously add new leaf-material to the top of the garden, while the used-up vegetative material and old fungus is removed from the bottom and placed into the dump. My finding that *Escovopsis* is the least prevalent in the top of gardens indicates that this pathogen persists in the older regions. This suggests an interesting host-parasite battle within individual gardens, with the parasite attempting to spread upward toward the fresh, and likely more nutrient rich, regions of the garden. In turn, the ants, which apparently can detect the presence of *Escovopsis* (Currie unpublished data), attempt to remove it from the bottom regions.

The discovery that *Escovopsis* is a specialized and virulent parasite of the symbiosis among fungus-growing ants, their fungi, and filamentous bacteria indicates that this mutualism is not inherently resistant to exploitation by unrelated parasites. This suggests that other highly evolved mutualisms are also exploited by organisms that are not 'cheaters', but instead whole new taxa. However, it appears that the ability of *Escovopsis* to exploit this mutualism results from the fact that it has co-evolved for much of the evolutionary history of this association (Currie unpublished data). In fact, the gardens could be inherently resistant to most alien fungi, especially in the derived clades, as extensive isolations revealed that only *Escovopsis* typically occurs in the garden (section 2.0 or Currie *et al.* 1999b). Apparently the combined defenses of the ants and their mutualistic microbes are enough to suppress most microbes, but are circumvented by the specialized parasite *Escovopsis*. Studies on other highly evolved mutualisms should look for the presence of unrelated taxa (especially microbes) exploiting these associations.

Table 3.1 Frequency of *Escovopsis* in colonies of leaf-cutting ant species from different location in the canal region of Panama (Barro Colorado and Fort Sherman Military Reservation, are abbreviated BCI and FSMR, respectively). Sampling of *A. colombica* colonies on BCI were conducted by sampling refuse material as it was removed from the colony. Sampling for the remaining species and locations was done by collecting garden material and plating pieces on nutrient agar.

Ant species	Gamboa	BCI	FSMR
<i>Acro. echinator</i>	75% (n=8)	-	-
<i>Acro. octospinosus</i>	68.4% (n=19)	-	-
<i>A. cephalotes</i>	50.0% (n=14)	-	18.2% (n=11)
<i>A. colombica</i>	48.5% (n=33)	66.0% (n = 53)	47% (n=19)

Figure 3.1. The relation between colony age and presence of the parasite *Escovopsis* in *Atta* spp. colonies. The age of each colony was estimated based on the number of openings and surface area (see materials and methods).

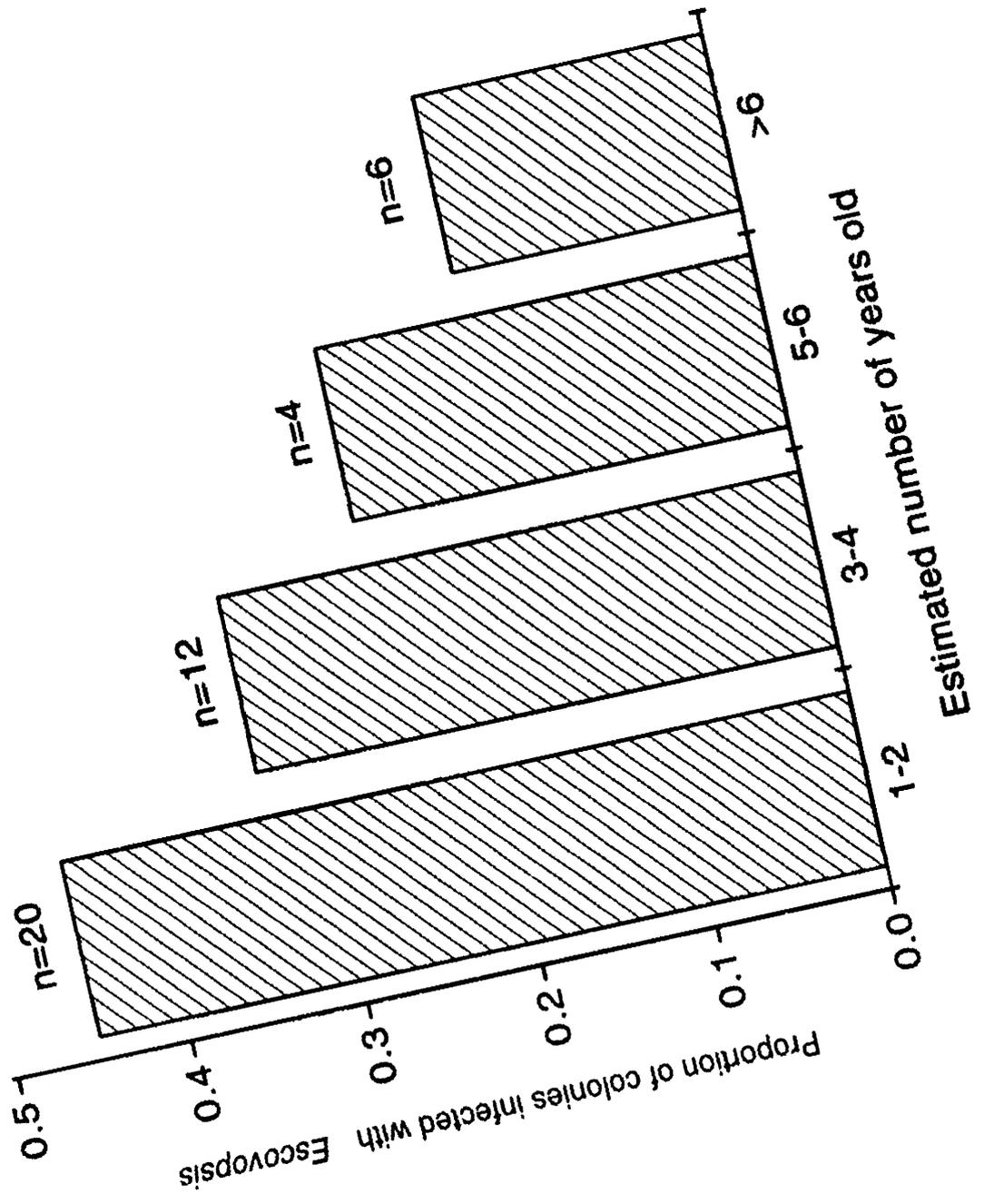
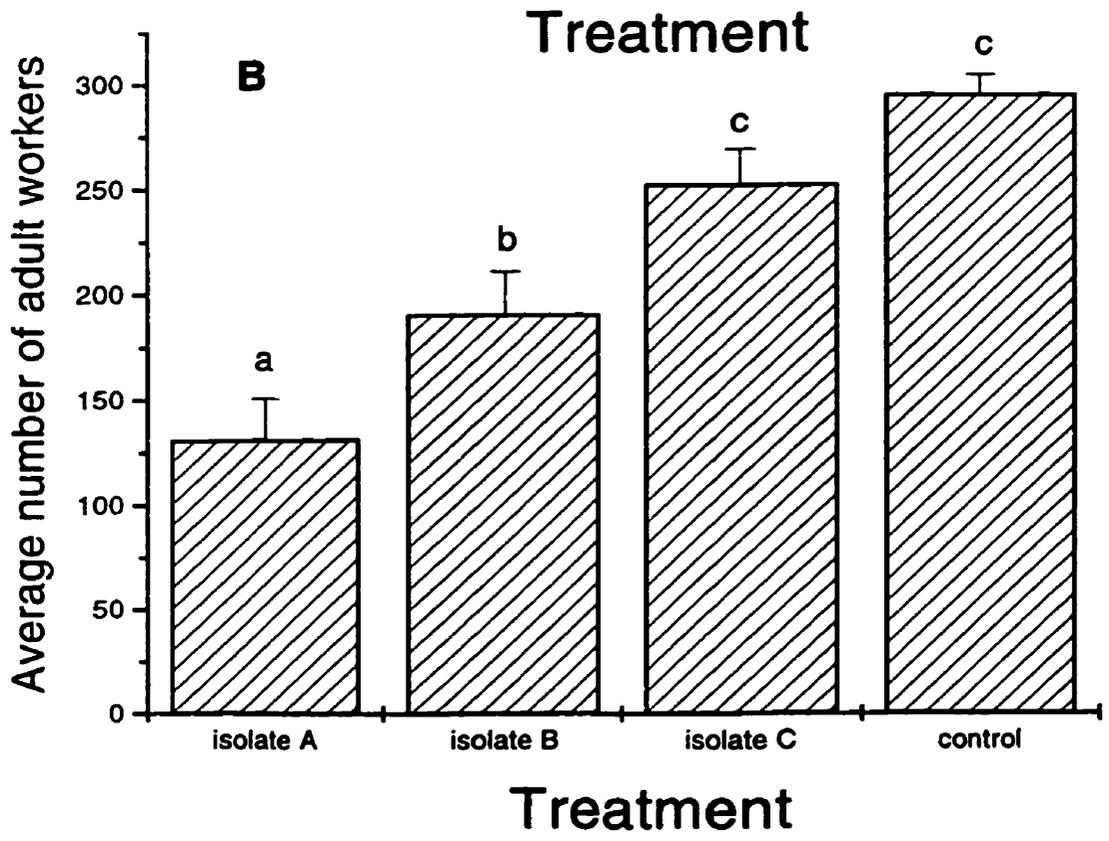
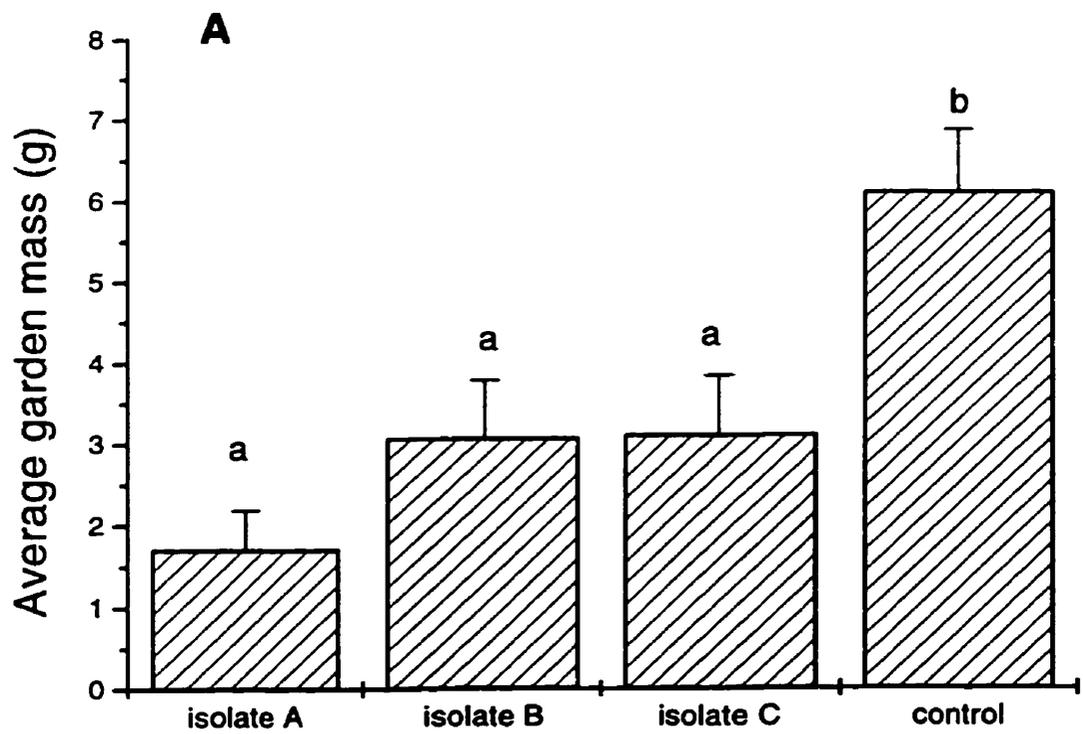


Figure 3.2. Impact of *Escovopsis* on the growth rate of *A. colombica* colonies. Three different isolates of *Escovopsis* obtained from *A. colombica* colonies within the same population were used to explore the possibility of differences in virulence. Each treatment was replicated 5 times using complete incipient *A. colombica* colonies, and differences were compared using an ANOVA (a, b, and c, represent significant differences in treatments). **A.** Difference in garden mass. Gardens infected with *Escovopsis* had significantly lower mass compared to uninfected colonies ($F=19.63$, $df=1, 14$, $P<0.0006$). **B.** Difference in number of workers. Infected gardens had significantly fewer workers than uninfected colonies ($F=27.65$, $df=1, 14$, $P<0.0001$). **C.** Difference in number of pupae. *Escovopsis* infected colonies had significantly fewer pupae than uninfected colonies ($F=24.52$, $df=1, 14$, $P<0.0004$). **D.** Difference in number of larvae. Colonies that were infected had significantly fewer larvae than uninfected colonies ($F=21.92$, $df=1, 14$ $P<0.0002$). (Error bars represent standard Errors).



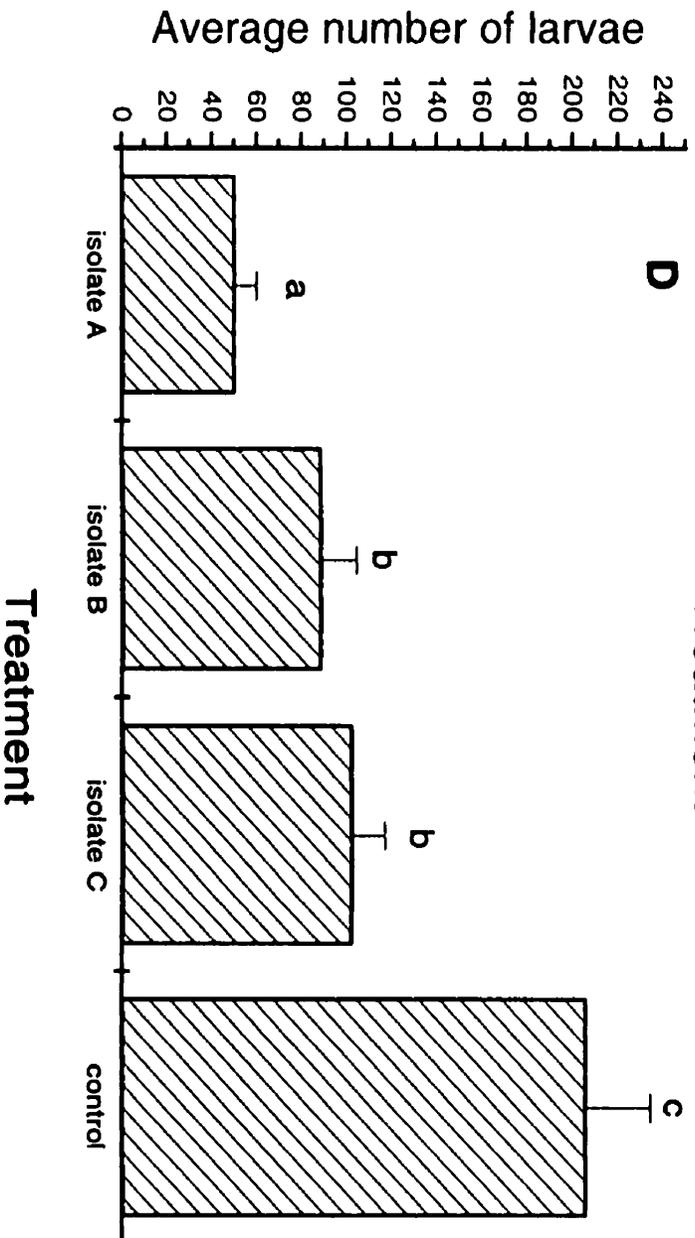
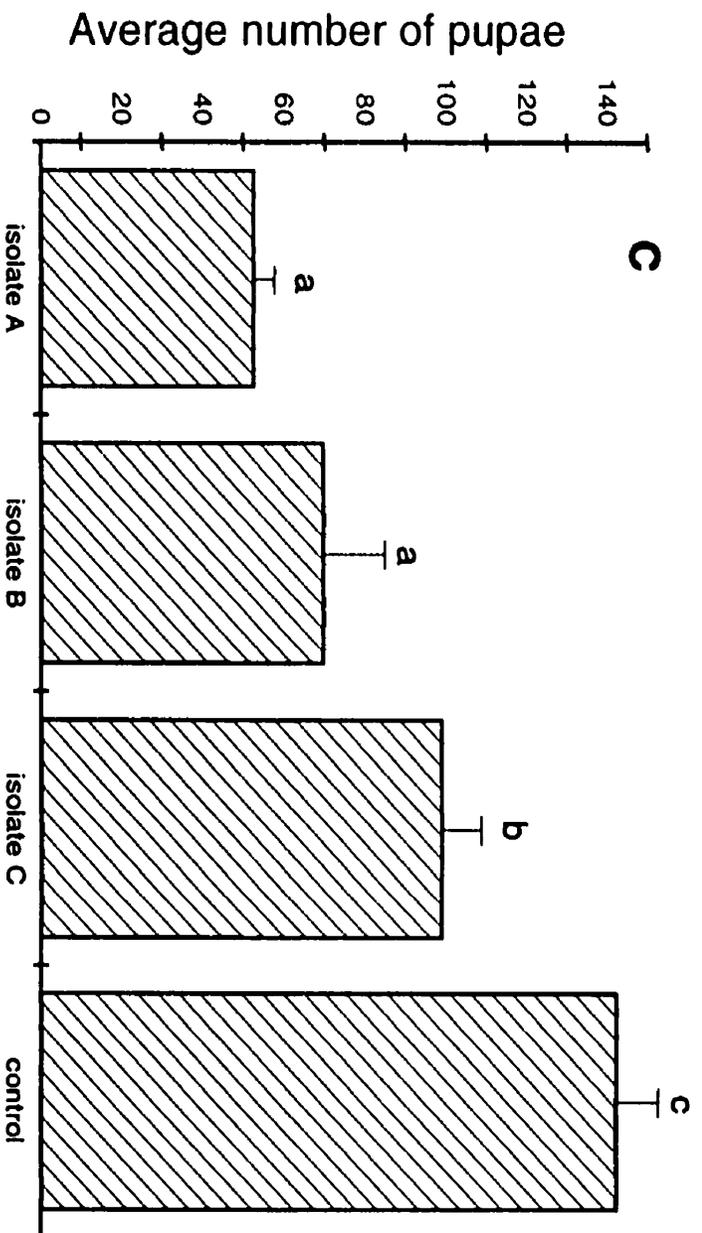
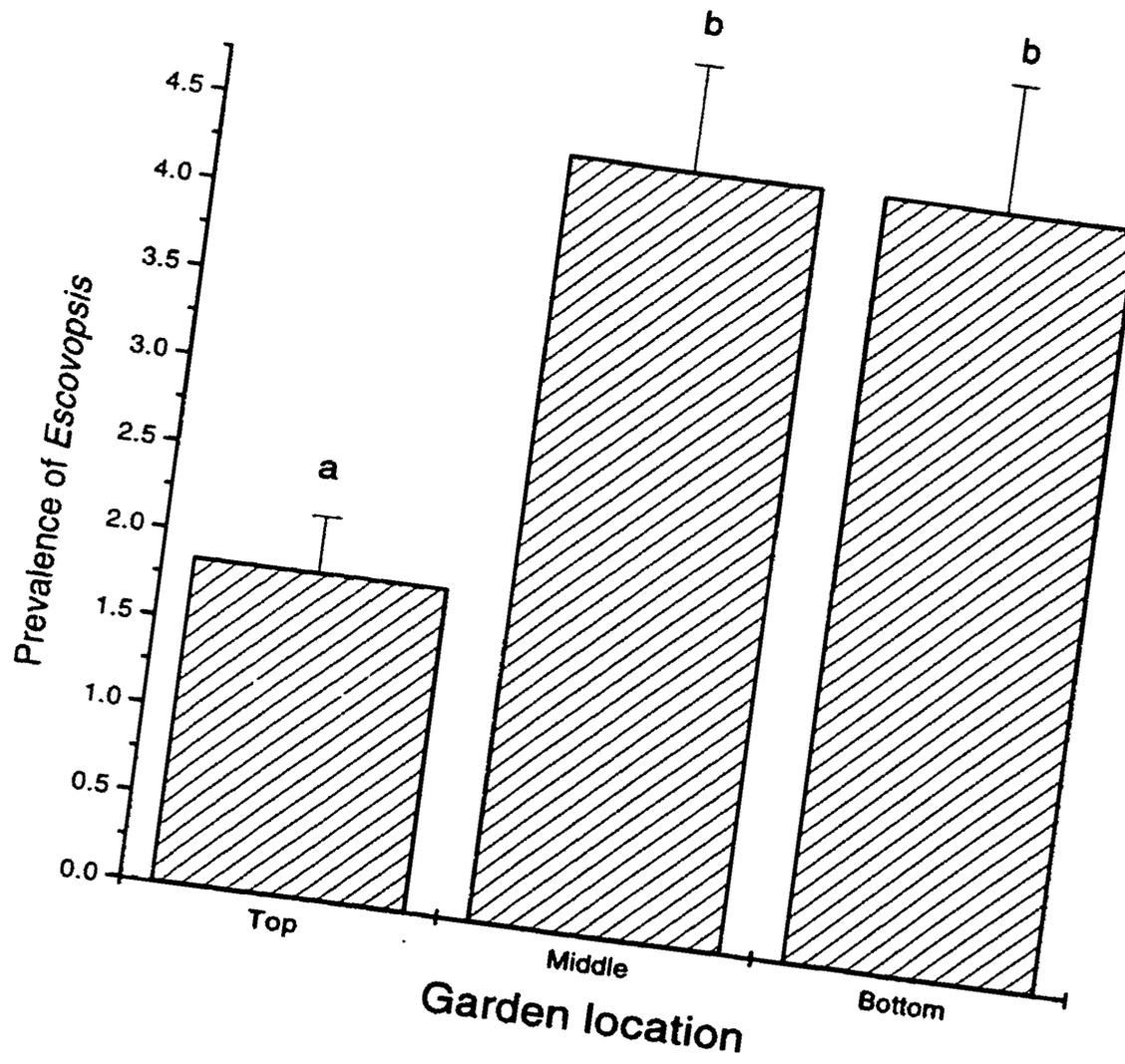


Figure 3.3. Prevalence of *Escovopsis* within different locations of the infected gardens of *A. colombica*. The upper, or younger, region of the garden had significantly lower prevalence of *Escovopsis* than the older regions ($n = 15$, $P < 0.05$ Tukey-Kramer HSD).



4.0 FUNGUS-GROWING ANTS USE ANTIBIOTIC PRODUCING BACTERIA TO CONTROL GARDEN PARASITES

(Previously published in *Nature*, **398**:701-704, with J.A. Scott, R.C. Summerbell, and D. Malloch)

4.1 ABSTRACT AND INTRODUCTION

The well studied, ancient, and highly evolved mutualism between fungus-growing ants and their fungi has become a model system in the study of symbiosis (Weber 1966, Wilson 1971, Chapela *et al.* 1994, Mueller *et al.* 1998, North *et al.* 1997). Although it is currently thought to involve only two symbionts associated in near isolation from other organisms (Weber 1966, Wilson 1971, Chapela *et al.* 1994, Mueller *et al.* 1998, North *et al.* 1997), recent work has established that the fungal gardens of attine ants are host to a specialized and virulent parasitic fungus in the genus *Escovopsis* (Ascomycota) (section 2.0 or Currie *et al.* 1999). Because the ants and their fungi are mutually dependent, the maintenance of stable fungal monocultures in the presence of weeds or parasites is critical to the survival of both organisms. Here I report on a new, third mutualist in this symbiosis, a filamentous bacterium (actinomycete) in the genus *Streptomyces* that produces antibiotics specifically targeted to suppress the growth of the specialized garden parasite *Escovopsis*. This third mutualist is associated with all species of fungus-growing ants examined, is carried upon regions of the ants' cuticle that are genus-specific, is transmitted vertically, and has the capacity to promote the growth of the fungal mutualist, indicating that the association of *Streptomyces* with attine ants is both highly evolved and of ancient origin.

4.2 RESULTS AND DISCUSSION

Because few organisms cultivate their own food, fungus-gardening by ants (Attini: Formicidae) is considered a major breakthrough in animal evolution (Wilson 1986). These ants forage on a diversity of substrates that they utilize for the cultivation of the vegetative mycelium of a fungus, their dominant food source. Fungus cultivation evolved apparently only once in the attines over 50 million years ago with the domestication of a fungus in the family Lepiotaceae (Agaricales: Basidiomycota) (Chapela *et al.* 1994, Mueller *et al.* 1998, Schultz and Meier 1995). Additional lepiotaceous lineages, and in one case a distantly related non-lepiotaceous basidiomycete, were domesticated in subsequent evolutionary history (Mueller *et al.* 1998). The success of fungal cultivation by the attine ants is illustrated by the leaf-cutting genera, *Acromyrmex* and *Atta*, which are the dominant herbivores in the Neotropics (Hölldobler and Wilson 1990).

Certain areas of the cuticle of fungus-growing ants are coated with what appears to the naked eye as a powdery, whitish-gray crust (Fig. 4.1). The few researchers who have noted it have dismissed it as a “waxy bloom”, implying that its etiology was as cuticular exudate (Weber 1972). Micromorphological and biochemical examination reveals that this coating is formed from masses of a filamentous bacterium (actinomycete) in the genus *Streptomyces* (Fig. 4.2a, see methods). Actinomycetes are mostly soil-dwelling organisms of great abundance and ecological importance that produce an array of secondary metabolites, many of which have specific antibacterial or antifungal properties (Waksman and Lechevalier 1962, Goodfellow and Cross 1984). In fact, the majority of antibiotics developed for human pharmaceuticals

are actinomycete metabolites, many derived from the genus *Streptomyces* (Waksman and Lechevalier 1962, Goodfellow and Cross 1984). In light of the unique biochemical properties of actinomycetes as a group, I hypothesized that the *Streptomyces* associated with fungus-growing ants serves an important role in this symbiosis by suppressing the growth of potentially devastating pathogens.

To ascertain the distribution and prevalence of this newly discovered bacterium, both within and between species and genera of fungus-growing ants, I examined 22 species of attine ants that represent 8 genera for the presence of the actinomycete. I found the bacterium to be associated with all species examined from the phylogenetically basal genera *Myrmicocrypta* and *Apterostigma* to the highly derived leaf-cutting genera *Acromyrmex* and *Atta*. All 112 colonies from Panama sampled for the presence of the actinomycete in 1997 and 1998 were found to have this bacterial association. In all cases, the actinomycete was concentrated on genus-specific areas of the ant integument that appear to be modified for the maintenance and growth of the *Streptomyces*, conceivably to facilitate the distribution of bacterial metabolites throughout the garden (Fig. 4.2, Table 4.1). In workers and queens of *Myrmicocrypta* and *Apterostigma* (phylogenetically basal genera) the actinomycete mutualist occurs under the forelegs (Fig. 4.2b); in the more phylogenetically derived genera (Table 4.1), the actinomycete mutualist is most prominent on the laterocervical plates of the propleura (Fig. 4.2c), ventral, collar-like structures immediately posterior to the mouthparts. The consistent association of the actinomycete with phylogenetically diverse attine ants, as well as its location on the ant integument (i.e. conserved within species but unique between genera), suggests that this association is highly evolved and may be of ancient origin.

The presence of the actinomycete on foundress queens (gynes) during their mating flights was studied in order to determine if, like the fungal mutualist, this bacterium is vertically transmitted between parent and offspring colonies. I examined 74 foundress queens and 15 males collected from throughout Gamboa, Panama, during the mating flight of *A. octospinosus* on May 9, 1997. *Streptomyces* was present on the cuticle of all gynes examined, whereas it was absent from all males collected during the same mating flight. Examination of female and male reproductives in natal gardens of *Trachymyrmex* cf. *zeteki* prior to mating flights revealed the exclusive presence of the cuticular actinomycete on females (n=74 ants, including 43 males, from 10 colonies). Because males do not participate in the founding of new colonies or in tending the fungal garden, these data support the hypothesized role of the actinomycete in suppressing the growth of garden pathogens.

To determine whether the attine-associated actinomycete produces compounds with beneficial antimicrobial properties, bioassays were conducted in which taxonomically diverse sets of fungi were challenged with attine *Streptomyces* isolates. *Streptomyces* isolates obtained from *A. octospinosus* lacked detectable inhibitory effects on the growth of generalist saprotrophic fungi, entomopathogenic fungi, and other fungi commonly used for antibiotic screening. However, it demonstrated potent inhibitory effects upon *Escovopsis*, a fungal genus (anamorphic Hypocreales: Ascomycota) (Seifert *et al.* 1995) recently identified as a specialized virulent parasite of attine fungal gardens (section 2.0 or Currie *et al.* 1999). The actinomycete completely suppressed spore germination of *Escovopsis* isolates in 25% of bioassays. Linear growth in the remaining challenges was inhibited by an average of

73.9±3.0% (mean ± standard error), typically resulting in zones of inhibition in excess of 30 mm (Fig. 4.3). Additional bioassay challenges between the corresponding *Streptomyces* and *Escovopsis* from colonies of *Cyphomyrmex longiscapus*, *Atta colombica*, and *Atta cephalotes* again resulted in significant inhibition of the growth of *Escovopsis*.

Growth enhancing effects by the actinomycete upon the basidiomycete mutualist were tested in broth culture bioassays using a *Streptomyces* isolate obtained from the phylogenetically basal genus, *Apterostigma*. Significant increases in basidiomycete vegetative biomass were observed in the presence of the actinomycete culture filtrate (e.g., averaging 47.9±7.6 mg vs. 5.3±2.4 mg dry weight for unamended controls, significant at $P = 0.0029$, t-Test, mean ± standard error). This result may be due to the production of growth promoting compounds by the actinomycete (e.g., vitamins, enzymes, and/or amino acids) (Martin and Martin 1970, Hervey *et al.* 1978, Cazin *et al.* 1989).

Various lines of evidence described above support the assertion that this newly discovered bacterial symbiont of the attine ants is a third mutualist in an ancient quadruple symbiosis among the ants, the domesticated fungi, the parasitic fungi, and the antibiotic producing bacteria. My conclusion is supported by the finding that this actinomycete: i) is associated with all attine species and all colonies intensively examined, representing the generic diversity of the Attini; ii) is vertically transmitted from parent to daughter nest, as is the fungal mutualist; and iii) promotes the growth of the fungal mutualist in vitro. More importantly, this *Streptomyces* iv) produces highly potent antibiotics that selectively inhibit the growth of the garden parasite *Escovopsis*. Although many antibiotics developed for

medicinal use have fairly broad-spectrum effects, it is likely that most fungicidal secondary metabolites produced by microbes evolved toward specific targets, such as competitors and pathogens (Vining 1990, Griffin 1994). Since the production of secondary metabolites is energetically costly and requires complex, genetically based biosynthetic pathways, its evolution and subsequent maintenance is presumed to impart a substantial selective advantage to the microbe (Vining 1990). Thus the production of antibiotics by the attine-associated *Streptomyces* that specifically target the growth of *Escovopsis* is compelling evidence that the bacterium is a highly evolved mutualist. The ants utilize this bacterium for its production of antibiotics to treat microbial infection of their garden, and in exchange, the ants disperse the actinomycete and appear to provide some form of nourishment for its growth.

While the ant-fungus mutualism is often regarded as one of the most fascinating examples of a highly evolved symbiosis, it is now clear that its complexity has been greatly underestimated. The attine symbiosis may be a co-evolutionary “arms race” between the garden parasite, *Escovopsis*, on the one hand, and the tripartite association amongst the actinomycete, the ant hosts, and the fungal mutualist on the other. The evolution of a mutualistic association between the attine ants and an actinomycete that suppresses parasites is perhaps not surprising. The benefits of such a symbiosis are illustrated by the paramount role of therapeutic antibiotics in recent human biomedical history. My findings suggest that microbes and their metabolites may be pivotal components in the regulation of other symbiotic associations between higher organisms, and thus a more detailed examination of their role promises to illuminate the general dynamics of symbiosis. Study of the presumably

intensively evolved chemical interactions between symbionts may provide valuable theoretical and practical insights regarding the identification, production, and application of antibiotics (Eisner 1990, Beattie 1992, Caporale 1995).

4.3 METHODS

4.3.1 Identification of bacterium

To identify the bacterium, micromorphological parameters as well as accepted biochemical criteria were used. A gas-liquid chromatographic analysis of the cell-wall fatty acid methyl esters was conducted (Holt *et al.* 1994). The absence of tuberculosteric acid and related 10-methyl esters excluded the genera *Actinomadura* and *Nocardiopsis*, which are morphologically similar to *Streptomyces*.

4.3.2 Attines examined

The presence of actinomycete was examined in fungus-growing ant species from the canal region of Panama (112 colonies, 17 species) and the Napo province of Ecuador (25 colonies, 5 species). Attine genera examined included a representative sampling of both “lower” (phylogenetically basal) and “higher” (phylogenetically derived) attines: *Myrmicocrypta* (2 species), *Apterostigma* (4 species), *Mycocepurus* (2 species), *Cyphomyrmex* (2 species), *Sericomyrmex* (1 species), *Trachymyrmex* (5 species), *Acromyrmex* (3 species), and *Atta* (3 species).

4.3.3 Antibiotic bioassay challenges

Bioassays were performed in Petri plates with an actinomycete isolate obtained from a phylogenetically derived attine species, *Acromyrmex octospinosus*. Saprotrophic fungi isolated from attine gardens, including taxa closely related to *Escovopsis*, were tested, as were generalist entomopathogens. Bioassays on a diverse representation of fungi used for general anti-fungal screening were also conducted. Finally, challenges against representative strains of the specialist garden parasite *Escovopsis* were conducted. Specifically, challenges were conducted against two strains of *Metarhizium anisopliae* and one strain of *Beauveria bassiana*, as well as against the following common species of microfungi: *Absidia* sp., *Ascobolus crenulatus*, *Aspergillus fumigatus*, *Coprinus patouillardii*, *Cryptococcus albidus*, *Drechslera biseptata*, *Exophiala spinifera*, *Fusarium oxysporum*, *Mucor pyriformis*, *Penicillium* sp., *Pythium aphanodermatum*, *Schizophyllum commune*, *Sordaria fimicola*, and *Trichoderma* sp.

Each *Streptomyces*-fungal challenge was replicated three times and conducted on Czapek Yeast Autolysate Agar. The actinomycete was inoculated on Petri plates and grown to a ca. 1.5 cm diameter before fungal strains were point-inoculated near the edge of the culture. Challenges were monitored every two days and growth inhibition of tested fungi scored as a reduction of growth rate, as compared to fungal cultures grown without the *Streptomyces*, or as complete suppression of growth. Bioassays for possible antibiotic production specific to the specialized parasite *Escovopsis* were conducted in the same manner as against other potential contaminants except that each challenge was replicated 5 times. Four strains of *Escovopsis* isolated from the gardens of different *A. octospinosus* colonies in Panama in 1997

were tested against *Streptomyces*. Production of antibiotics specific toward *Escovopsis* was examined in additional species of attines, including *C. longiscapus*, *A. colombica*, and *A. cephalotes*. The presence of a zone of inhibition in bioassays indicates (i) the production of diffusible metabolites by the actinomycete, and (ii) the susceptibility of the test fungus to these compounds. Since inhibition is dose dependent, the detection of partial inhibition implies the existence of a dose that would impart complete inhibition.

4.3.4 Growth promotion bioassays

Broth cultures of the attine-fungus isolated from an *Apterostigma* colony were grown with extract from the *Streptomyces* isolated from this species. Actinomycete extract was obtained by growing *Streptomyces* in Czapek Yeast Autolysate broth for two weeks and then passing the broth through a low protein binding sterilizing filter unit (Millipore, Millex, GV) to remove bacterial biomass. Each bioassay was carried out in 5 replicates and was conducted in 50 ml of Czapek Yeast Autolysate broth.

Table 4.1 Location of *Streptomyces* on different genera of attine ants

Attine genera*	Under forelegs On propleura	laterocervical plate of the propleura
<i>Myrmicocrypta</i>	X	
<i>Apterostigma</i>	X	
<i>Mycocepurus</i>	X	X
<i>Cyphomyrmex</i>		X
<i>Trachymyrmex</i>		X
<i>Acromyrmex</i>		X
<i>Atta</i> †	not visible on exoskeleton	

* Attine genera listed from most phylogenetically basal to most phylogenetically derived (Schultz and Meier 1995, Wetterer *et al.* 1998).

† *Streptomyces* repeatedly isolated from intact *Atta* spp.

Figure 4.1 Photograph illustrating the presence of the third mutualist, *Streptomyces*, on the cuticle of *A. octospinosus*.

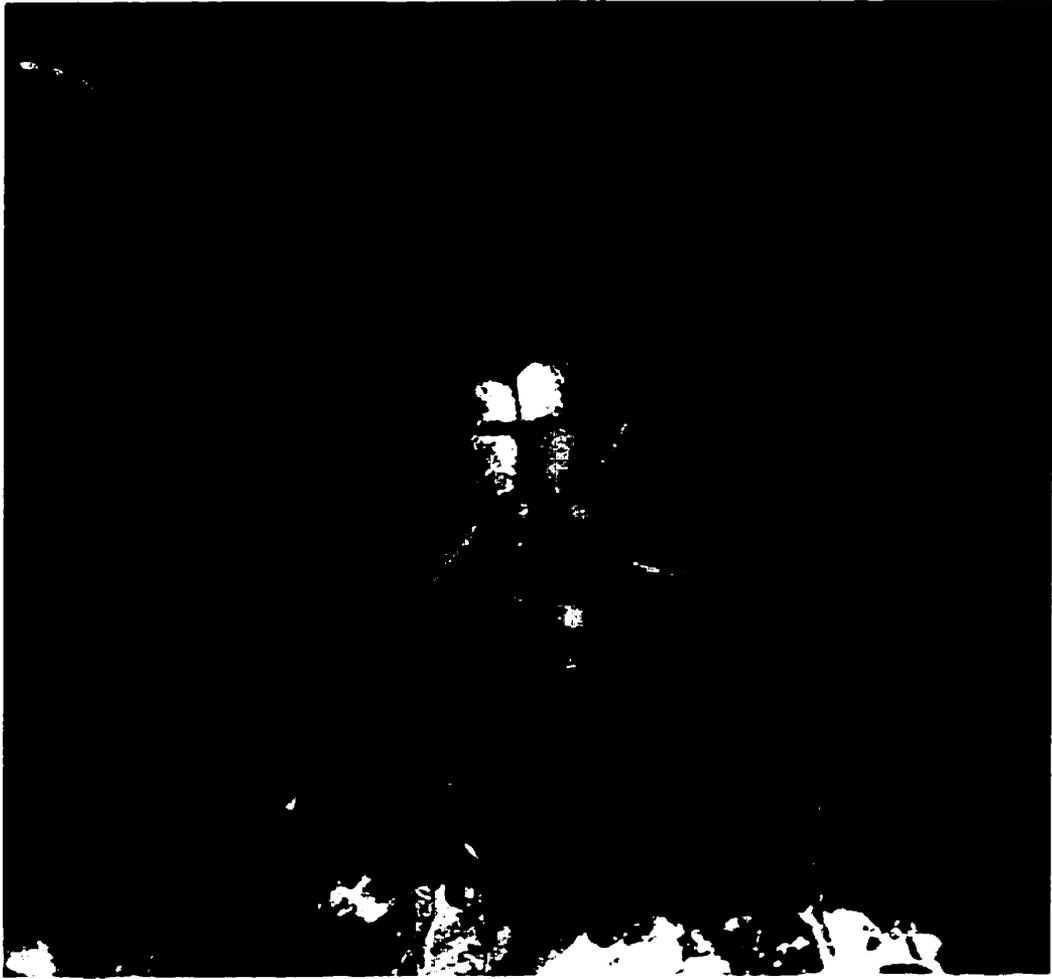


Figure 4.2 Scanning electron micrographs of fungus-growing ants showing the location of *Streptomyces*. **a**, View of the filamentous growth form of the actinomycetous bacterium, demonstrating typical growth pattern and thickness on the cuticle. Scale bar represents 10 μm . **b**, View of *Streptomyces* under the forelegs (arrow) of *Apterostigma* sp., characteristic location of the bacterium in phylogenetically basal genera of the Attini. Scale bar represents 100 μm . **c**, Ventral view of a minimum worker of *A. octospinosus*. The actinomycete laden laterocervical plate (arrow) can be seen just below the head on the propleura of the ant. This is the characteristic location of the bacterium on the more phylogenetically derived genera of the Attini. Scale bar represents 500 μm .

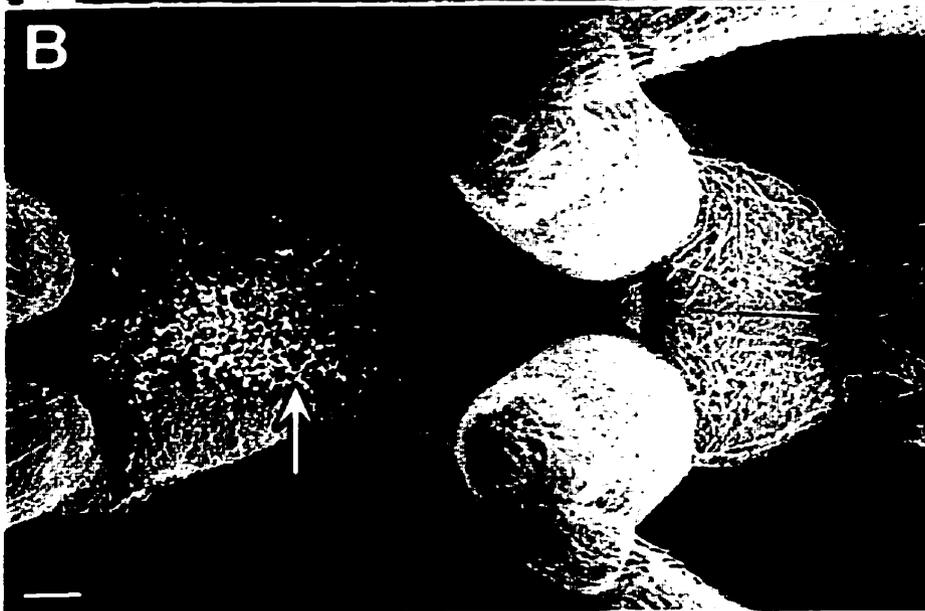
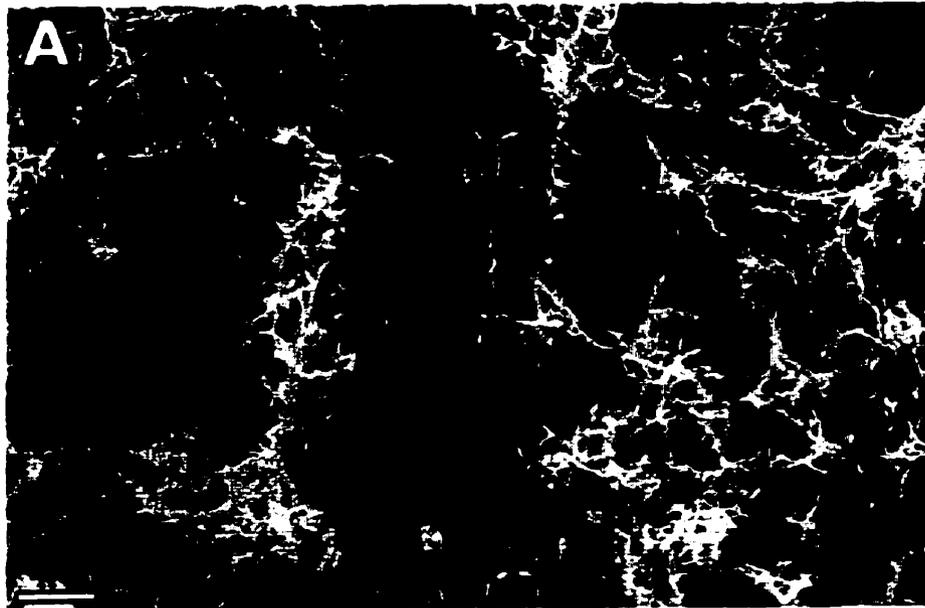
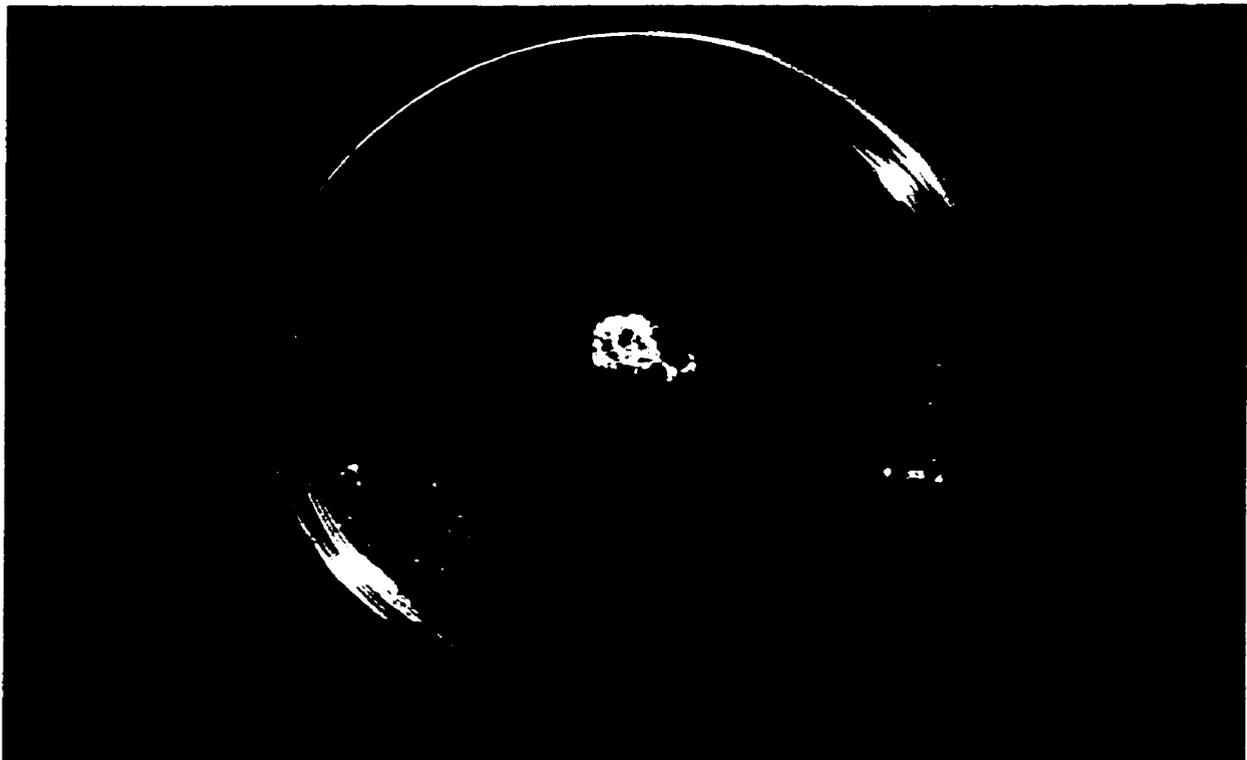


Figure 4.3 Bioassay challenge between *Streptomyces* and *Escovopsis*, the specialized parasite of attine fungal gardens, associated with *A. octospinosus* illustrating the substantial zone of inhibition.



5.0 EXPERIMENTAL EVIDENCE OF A TRIPARTITE MUTUALISM: BACTERIA HELP PROTECT LEAF-CUTTING ANT FUNGAL GARDENS FROM SPECIALIZED PARASITES

5.1 ABSTRACT

This study examines the interactions among leaf-cutting ants, their symbiotic filamentous bacteria (actinomycetes), and a specialized microfungal parasite (*Escovopsis*) in their fungal gardens. To examine the role of the actinomycete (which in most species is present on the ants' integument) in suppressing the growth of *Escovopsis* within leaf-cutting gardens, a two-by-two factorial design experiment was conducted, crossing the presence/absence of actinomycete with the presence/absence of *Escovopsis*. In the first experiment, infection by *Escovopsis* became much more extensive within fungal gardens with the bacterium removed from workers, compared to gardens with the bacterium still present on the ants. In a second experiment, the presence of *Escovopsis* had a significant negative impact on the size of gardens. However, in the presence of the specialized parasite *Escovopsis*, gardens maintained by workers with the actinomycete were significantly larger than those tended by actinomycete-free workers. In the absence of the parasite, no significant difference between sub-colonies with and without the actinomycete was observed.

Leaf-cutting ants have a complex social structure, with a division of labor based on the size and age of individual workers that is typically optimized for the tasks performed. If the bacterium is used by the ants to help maintain the health of the garden, one may predict that the greatest biomass of actinomycete is carried by workers whose task it is to tend the

garden. In my study, garden-tending workers carried significantly more actinomycete on their integument than foragers. However, in a comparison of the two distinct size castes of garden-tending individuals, minimum workers had lower total biomass of actinomycete than major workers. This suggests that the increased surface area of larger workers may be more effective for maintenance of the actinomycete.

A final experiment examined the possibility of pathogen-induced increases in actinomycete abundance on workers. A significantly higher abundance of actinomycete was found on workers in colonies experimentally infected with *Escovopsis* as compared to uninfected control colonies. My experiment suggests that the ants are able to regulate actinomycete growth and promote the growth of the bacterium when colonies are stressed, possibly through glandular secretions that provide nutrients for the bacterium. I suggest that symbiont-mediated reductions in the impact of parasites may be an important, but greatly overlooked, aspect in the cost-benefit assessment of mutualism and, as in the mutualism between fungus-growing ants and their fungi, may in some situations lead to the evolution of additional mutualists within symbioses.

5.2 INTRODUCTION

The importance of mutualism in shaping communities and ecosystems, especially tropical ones, is widely recognized (Boucher 1985, Margulis and Fester 1991, Douglas 1994).

However, the evolutionary origins of mutualisms and their long-term stability are a challenge to evolutionary theory. One current theory proposes that mutualistic cooperation evolves from a parasitic association that gradually becomes less exploitative (e.g., commensalism)

and may eventually evolve into a form of mutually beneficial symbiosis (Axelrod and Hamilton 1981, Bull and Rice 1991). This theoretical position has been extended into a belief that mutualism is less of a partnership than a mutual exploitation (Nowak *et al.* 1994, Maynard Smith and Szathmary 1995, Leigh and Rowell 1995, Herre *et al.* 1999), indicating that determining the costs and benefits between symbionts, and those factors that influence them, is fundamental to the study of mutualism (Cushman and Beattie 1991, Bronstein 1994, Herre and West 1997, Herre *et al.* 1999). Studies of mutualistic interactions typically are framed with the view that these symbiotic associations are bipartite systems, with two symbionts interacting largely in isolation from other organisms. However, interspecific interactions of mutualists with non-mutualists or even non-symbionts may alter the costs and benefits between the mutualists (e.g., Gehring and Whitham 1991, Sanders *et al.* 1993, Guame *et al.* 1998, Yu and Pierce 1998, Gastreich 1997). Although the mutualism between fungus-growing ants and their fungi traditionally has been thought to involve only two symbionts associated in near isolation from other organisms (Weber 1966, Weber 1972, Wilson 1971, North *et al.* 1997), recent work has suggested the presence of both a third mutualist and a highly evolved parasite in the mutualism (see sections 2.0 or Currie *et al.* 1999b, section 3.0, section 4.0 or Currie *et al.* 1999a). Here I experimentally explore the importance of this alleged third mutualist, and use this model system in the study of symbiosis to illustrate the importance of supernumerary components in mutualisms in general.

Fungus-growing ants (Attini: Formicidae: Hymenoptera) have an ancient and obligate association with fungi that originated perhaps as long as 65 million years ago (Wilson 1971,

Mueller *et al.* submitted). The fungi, mostly belonging to the family *Lepiotaceae* (Agaricales: Basidiomycota) (Chapela *et al.* 1994, Mueller *et al.* 1998), are cultivated by the ants and, in exchange, serve as their main source of food. Although the 12 genera of fungus-growing ants use a diversity of substrates for growing their cultivars, the phylogenetically derived sister genera *Acromyrmex* and *Atta* (Schultz and Meier 1995, Wetterer *et al.* 1998), commonly known as leaf-cutter ants, are specialized to use mostly fresh leaves and flowers. New leaf-cutting ant queens carry an inoculum of fungal material from their parent colony during their mating flight, and thus clonally propagate the fungus from one generation to the next.

The gardens of fungus-growing ants are frequently infected with a specialized and virulent parasitic fungus in the genus *Escovopsis* (section 2.0 or Currie *et al.* 1999b). *Escovopsis* is a genus of microfungi that is allied with the Ascomycota order Hypocreales, although no teleomorph has yet been identified (Currie unpublished data). The only habitat that *Escovopsis* has been isolated from is the gardens and dumps of attine ants (Seifert *et al.* 1995, section 2.0 or Currie *et al.* 1999b, Bot *et al.* submitted). *Escovopsis* has a major impact on the health of the garden; even in the presence of the ants it can overwhelm the ants' gardens, killing the colony (Möller 1893, section 2.0 or Currie *et al.* 1999b). In addition, *Escovopsis* can maintain a continuous presence within colonies once infection has become established, which results in dramatic decreases in the growth rate of the colony (section 2.0 or Currie *et al.* 1999b, section 3.0). Since colonies must reach a minimum size before reproduction is possible, this parasite can have dramatic effects on the fitness of leaf-cutting ants (see section 3.0). The long history of clonal propagation of the fungal cultivars by leaf-cutting ants has

also apparently resulted in higher susceptibility to the pathogen *Escovopsis* (section 2.0 or Currie *et al.* 1999b), perhaps a result of the lower genetic diversity of the fungus, the longer evolutionary adaptation of the pathogens to the cultivars, or both.

Fungus-growing ants have a fourth symbiont, a filamentous bacterium (actinomycete), currently classified in the genus *Streptomyces* (section 4.0 or Currie *et al.* 1999a). The actinomycete is associated with all species of fungus-growing ants examined, is carried, in some taxa, upon modified regions of the ants' integument, and is localized on genus-specific areas of the ants' cuticle. In addition, this bacterium is vertically transmitted as it is carried by the gynes (female reproductive ants) on their mating flight, transferring the bacterium from parent to offspring colony. Although it appears that this bacterium has the capacity to promote the growth of the cultivated fungi among at least some lower attines, the main benefit that the bacterium provides to fungus-growing ants and their fungi is likely the production of antibiotics that suppress the virulent parasite *Escovopsis* (section 4.0 or Currie *et al.* 1999a). Extensive bioassays established that these actinomycetes produce secondary metabolites that have potent inhibitory properties against *Escovopsis* spp., but do not inhibit other fungi. Since these findings were obtained *in vitro*, experimental evidence indicating that the antibiotics produced by the attine ant-associated actinomycete suppresses the growth of *Escovopsis* within gardens is still needed. In the present study I experimentally investigate the role of the actinomycete in dealing with *Escovopsis* infection in gardens of the leaf-cutting ant *Acromyrmex octospinosus*. In addition, I test whether the actinomycete bloom is more prevalent on individual workers specialized on tending the garden, as would be expected if it is important for maintaining the health of the fungal gardens. Finally, the

possible role of ants in supporting the growth of the actinomycete on their exoskeleton is investigated by determining whether experimental infection of previously unparasitized colonies with *Escovopsis* influences the abundance of the bacteria on garden-tending individuals. The adaptive significance of symbionts ameliorating the impact of parasites on their hosts is discussed.

5.3 METHODS

5.3.1 Study organisms

Interspecific interactions among fungus-growing ants, symbiotic actinomycetes, and the garden parasite *Escovopsis* were examined using colonies of the leaf-cutter ant *Acromyrmex octospinosus*. This species forages on the flowers and leaf material of a variety of different plant species, which it uses to grow its fungal gardens. *Acromyrmex octospinosus* is abundant and widely distributed throughout the neotropics, occurring throughout most of South and Central America (Weber 1972). Mature colonies are composed of thousands of workers, with perhaps as many as 35,000 individuals (Weber 1966, Wetterer 1999) and a single queen. *Acromyrmex* workers have a size polymorphism that is bimodal with minor and major workers (Bot and Boomsma 1996, Wetterer 1999). The minor workers are a specialized caste that tend the garden while the major workers engage in both garden-tending tasks and foraging for vegetative material. In addition, *Acromyrmex* species have a strong age-based division of labor, especially in the major worker caste, with young major workers tending the garden, older ones foraging for substrate, and the oldest workers managing the dump (Hölldobler and Wilson 1990, Hart and Ratnieks submitted). In the leaf-cutting ant genus *Acromyrmex*, the actinomycete is most concentrated on the laterocervical plates, which

are collar-like structures immediately posterior to the mouth-parts on the ventral surface of the ants' thorax. However, the bacterial growth frequently completely covers the ants' exoskeleton as a grayish-white bloom, giving the individual a white appearance that contrasts with the typical reddish to dark-brown color of the ants.

The colonies used in this experiment were collected in Gamboa, Panama in 1996 and 1998. Prior to the experiment, colonies were kept in plastic boxes (32 cm by 22 cm), with one fungus garden (ca. Volume of 1 L) under a plastic beaker and covered to exclude light. The experiments were conducted in a climate room at the University of Aarhus in Denmark (25° C, RH = 60-70%) and, unless otherwise noted, colonies were fed bramble leaves (*Rubus* sp.) three times a week. To prevent ants from escaping, fluon (Northern Products, Inc., Rhode Island) was applied to the sides of the colony chambers.

5.3.2 Experimental tests of actinomycete suppression of *Escovopsis*

Two possible benefits of the actinomycete for fungus-growing ants and their fungi were examined. I explored the role of the bacterium in: i) reducing the prevalence of *Escovopsis* within gardens and ii) decreasing the negative impact of *Escovopsis* on the health of *A. octospinosus* fungal gardens (measured as garden size). It was necessary to conduct two separate experiments because of the difficulty in studying both factors within the same experimental setup (see below).

In both experiments I used a two-by-two factorial design that crossed the presence/absence of *Escovopsis* (garden experimentally infected versus not infected) with the presence/absence of

the actinomycete (removed from workers versus not removed). Infections of the parasite *Escovopsis* were achieved by spraying sub-colonies with ca. 50-100,000 spores suspended in sterile, distilled water. To disperse the spores evenly within the water, 1-2 $\mu\text{l/ml}$ of the wetting agent Tween 20 was added. As a control, colonies not receiving *Escovopsis* treatment were sham-sprayed with sterile distilled water with 1-2 $\mu\text{l/ml}$ of Tween 20. Gardens were sprayed using a mist inoculator. To remove the actinomycete from the exoskeleton of workers, each individual was washed in an antibacterial solution (Penicillin G and Streptomycin sulfate, at 311 mg/L and 641.5 mg/L, respectively) for 30 seconds. In addition, any location with a concentration of the actinomycete, such as the laterocervical plates, was scraped with stiff forceps to ensure maximum removal of the bacterium from the ants. Workers from sub-colonies that were to retain the actinomycete were washed in sterile water, and a location without a concentration of actinomycete sham-scraped with stiff forceps.

In the first experiment, I examined the importance of the ant-associated actinomycete in decreasing *Escovopsis* prevalence within gardens. Large sub-colonies were used to increase the opportunity for infection either to spread throughout the garden or be contained (suppressed) by the ants through the application of the actinomycete metabolites. Large sub-colonies were also used to ensure the survival of the sub-colony throughout the duration of the experimental period (two weeks). Large sub-colonies were created by dividing five intact colonies each into four parts of approximately 4.0 g garden mass and 25-30 workers. Sub-colonies were placed in individual chambers with an inner circular container (height 3 cm, diameter 7.8 cm) for the garden and an outer chamber (height 6 cm, length 17 cm, width 11

cm) for foraging and dumping of refuse material. Each sub-colony was randomly assigned to one of the four treatment combinations, with five replicates (across colonies) of each treatment combination.

After receiving treatments, colonies were maintained in the climate room for 14 days. At the end of the experimental period, 18 small individual pieces of garden material (ca. 4 mm³) were isolated onto nutrient agar. Aseptic isolations were done on potato dextrose agar medium (Difco Lab., Detroit, Michigan) with antibacterial antibiotics (Penicillin and Streptomycin sulfate at 50 mg/L each). Each inoculum was monitored daily and the presence of *Escovopsis* growth noted.

The prevalence of *Escovopsis* within gardens was statistically compared using a logistic analysis (JMP). Also, the garden mass in these large sub-colonies was compared using an ANOVA, with the initial and final mass used as a repeated measure (SYSTAT). However, mass changes in these sub-colonies are greatly influenced by the absence or presence of new leaf material being added to the garden. In my study, only some sub-colonies added new leaf material to the garden, irrespective of treatments received, so changes in garden mass in this experimental setup are not very meaningful.

In the second experiment, I examined the impact of the interaction between the actinomycete and the fungal parasite *Escovopsis* on garden mass using small sub-colonies. Since these small sub-colonies never add leaf material to the garden because of their limited garden size and presence of few workers, they allowed us to compare the garden mass between

treatments without the confounding factor of the addition of new leaf material to the garden (as occurs in the large sub-colonies, see above). In addition, the use of small sub-colonies allowed an increase in sample replicates, thus increasing the power of the statistical comparison.

Each sub-colony consisted of four minor and major workers. A single worker pupa was added to each sub-colony, to promote normal behaviour in workers. The initial wet garden mass was ca. 80 mg. Moist tissue paper was placed at the bottom of containers (diameter 2.7 cm, height 4 cm) with a small bramble leaf on top. The garden material and workers were placed on the top of the leaf, preventing the direct contact of the fungus with the moist paper (see Bot *et al.* in preparation, for further details regarding the small sub-colony setup). After two days, the fungus was weighed, and both garden and ants were placed in clean vials with a fresh leaf. Final fungus mass was measured 4 days after the beginning of the experiment, thus measures were obtained on experimental days 0, 2, and 4. The experiment was run for four days because these small sub-colonies remain stable for less than a week since new leaf material is not added to the garden. *Escovopsis* and actinomycete treatments were applied in the same fashion as described above for the first experiment. Three parent colonies were used, and each treatment was replicated 12 times within each of these colonies. Colonies were set up on different days, with colony replicates being blocked within a single day, in order to allow sufficient time to set up all 48 sub-colonies created from each parent colony. Statistical comparisons were performed using an ANOVA, with date of mass determination (0, 2, and 4) as a repeated measure.

5.3.3 Abundance of actinomycete on gardening versus foraging workers

To investigate the hypothesis that selection on leaf-cutting ants optimized the use of actinomycete for maintaining the health of the garden, the abundance of the bacterium was compared between different castes. If maintenance of actinomycetes is costly (e.g., because of costly supply of nutrients) and workers can facultatively adjust actinomycete growth rates, then workers specialized on garden maintenance may have a higher abundance of actinomycete as compared to foragers (i.e., those not engaged in garden maintenance). I tested this hypothesis using three intact colonies of *A. octospinosus*. Each colony was broken down into two sections: inside (predominately workers involved in garden maintenance) and outside (predominately foraging workers) of the garden chamber. Individuals working in the dump were not included in this comparison. Workers outside in the foraging chamber were collected first, to ensure that mixing of the two groups did not occur when disrupting the colony. Using a dissecting microscope, the abundance of actinomycete was categorized into two very distinct and conservative groupings: i) none present or small amount present, but only on the laterocervical plates of the propleura, or ii) a thick growth on the laterocervical plates, including other locations on the body, and frequently completely covering most of the body of the ant (including a majority of the dorsal and ventral surfaces of the head, thorax, and abdomen). First, I examined the differences in actinomycete abundance between the distinct size classes of the ants, comparing minor versus majors workers. Second, the abundance of actinomycete on major workers inside the garden chamber (i.e., young individuals that are involved in garden-tending tasks) were compared with major workers outside the garden chamber (i.e., older individuals involved in foraging for vegetative

substrate). Since minor workers do not forage, a comparison between nest locations was not made for this size caste. The two groupings of actinomycete biomass on workers were used as the dependent variable in a logistical analysis comparing position of workers (inside or outside the garden chamber).

5.3.4 Pathogen-induced actinomycete growth promotion

I conducted a final experiment to examine the potential for pathogen-induced growth promotion of the actinomycete on *A. octospinosus* workers. The abundance of bacteria on major workers was assessed for three colonies of *A. octospinosus*. The amount of actinomycete, using the same two broad categories defined above, was determined for each major worker. Two sub-colonies were created from each parent colony of *A. octospinosus* by removing ca. 4 g of garden and half of the workers divided evenly between the two sub-colonies. As in the first experiment, the sub-colonies were maintained in a two chamber system with the garden in a circular container (height 3 cm, diameter 7.8 cm) situated within an outer chamber for foraging (height 6 cm, length 22 cm, width 17 cm). The sub-colonies were allowed to stabilize their gardens for three days in the laboratory while being fed bramble leaves. Half of the colonies (one randomly selected sub-colony of each pair from the intact colonies) were stressed by spraying the garden with ca. 50-100,000 spores of *Escovopsis* dissolved in sterile distilled water using a mist inoculator (see details of the spraying methods above). Control colonies were sprayed with sterile water. The experimental colonies were maintained in the laboratory for 8 days before being broken down to determine the abundance of actinomycete. The abundance of actinomycete on workers between infected and uninfected colonies was compared using a logistic analysis.

5.4 RESULTS

5.4.1 Experimental tests of actinomycete suppression of *Escovopsis*

In the first experiment, the prevalence of *Escovopsis* was significantly lower in sub-colonies with the actinomycete present as compared to sub-colonies with the bacterium removed (Fig. 5.1a; $X^2=30.24$, $df=1$, $P<0.0001$). In this experiment, no significant difference in final garden mass was observed due to either the presence/absence of *Escovopsis* or presence/absence of actinomycete (Fig. 5.1b; $F_{1,16}=0.0$, $P<0.99$; $F_{1,16}=0.1587$, $P<0.6956$; respectively). As noted above (see methods), only some large sub-colonies add new leaf-material to the garden, irrespective of treatment received, and the addition of new leaf-material has a major impact on garden mass. Therefore, as anticipated *a priori*, I found no effect on garden mass from either treatment condition.

In the second experiment, a significant interaction between the presence/absence of actinomycete and the presence/absence of *Escovopsis* was obtained. More specifically, the gardens of sub-colonies with actinomycete-covered workers were on average significantly larger at the end of the experimental period when infected by the parasite, compared to sub-colonies with workers without the actinomycete (Fig. 5.2; $F=5.72$, $df=1$, $P<0.0204$).

5.4.2 Abundance of actinomycete on gardening versus foraging workers

I found that different worker castes had different abundance of actinomycete on their integument. The minor workers typically had actinomycete only on the laterocervical plates of the propleura, while major worker and female reproductive alates are frequently

completely covered with the bacterium (Table 5.1). Major workers within the garden chamber had significantly more actinomycete on their integument compared to major workers foraging for leaf material (Fig. 5.3; $X^2=82.00$, $df=1$, $P<0.0001$). More specifically, major workers tending the garden were often completely covered in actinomycete, while foragers usually had no actinomycete or just a small amount on the laterocervical plates.

5.4.3 Pathogen-induced actinomycete growth promotion

An increase in abundance of actinomycete was observed on major workers when colonies were stressed by infecting them with *Escovopsis* (Fig. 5.4). At the end of the experimental period, more workers in the infected colonies had a heavy covering of actinomycete on their integument as compared to sub-colonies not sprayed with *Escovopsis* ($X^2=8.24$, $df=1$, $P<0.0043$).

5.5 DISCUSSION

The results of this study clearly show that the symbiotic filamentous bacteria present on attine ants is a third mutualist modulating the mutualism between leaf-cutting ants and their fungal cultivars. I (section 4.0 or Currie *et al.* 1999a) showed that the actinomycete is vertically transmitted between parent and offspring colonies, a typical mode of transmission in mutualistic symbioses (Bull *et al.* 1991), and that the actinomycete produced antibiotics *in vitro* that specifically target the pathogen *Escovopsis*. In addition, the bacterium is associated with ants representative of the generic diversity of the attines, is most concentrated in specialized locations on the integument of workers, and its location differs across attine genera (section 4.0 or Currie *et al.* 1999a). My study provides experimental evidence within

colonies that the bacterium helps suppress the parasite *Escovopsis*. Specifically, I found both the prevalence of *Escovopsis* infection and the rate of garden loss to be significantly lower in the sub-colonies with the actinomycete present as compared to sub-colonies with the actinomycete experimentally removed.

Despite the removal of the actinomycete from ants, gardens were not completely devastated by *Escovopsis* in my study. However, this does not detract from the importance of the actinomycete in helping maintain the health of the garden. It is possible that some bacterium was still present after my actinomycete removal treatment, either in the garden or on workers, at an abundance below detectable levels. In addition, secondary metabolites secreted by the bacterium onto the garden prior to the experimental setup would likely still be present in the actinomycete removal treatments, at least early in the experimental period, and thus provide some temporary inhibition of *Escovopsis*. Finally, greater devastation of the garden in the absence of the actinomycete was likely diverted by the other defense mechanisms that are employed by the ants. For example, the ants physically remove the fungus *Escovopsis* through weeding and fungal grooming behaviors (Möller 1893, Weber 1972, Currie and Stuart in preparation). Nevertheless, I obtained a significant effect even over a short time period. Leaf-cutting ant colonies must survive for several years and grow to large sizes to reach sexual maturity, so any immediate marginal benefit would likely result in a huge advantage over the 10 or more year life span of a colony (Weber 1966). Although it would be valuable to conduct these experiments over a longer period, it would be extremely difficult. Only larger colonies with queens are sufficiently stable for long-term experiments, and in such set ups it would be difficult to remove a significant proportion of the

actinomycete as well as ensure the it does not grow back on cleaned or newly emerging workers.

Leaf-cutting ants have one of the most complex caste systems in social insects (Wilson 1980a, Wilson 1980b), with tasks performed by workers being correlated to the individuals' age and physical size. In the genus *Atta*, as many as 29 distinct tasks have been identified that are performed by, at the very least, 7 different castes, with each of these castes having higher or lower efficiencies at the different tasks (Wilson 1980a, Wilson 1980b). Such fine-grained division of labor probably optimizes ergonomic efficiencies. My study shows that the symbiotic association between the leaf-cutting ant *A. octospinosus* and their symbiotic actinomycete bacteria has been optimized within colonies for the purpose of garden maintenance. Foundress queens and young major workers who tend the fungal gardens typically are covered completely in the actinomycete, while the older major workers which forage for vegetation have almost no bacteria on their exoskeletons. These differences also are true in field colonies of both *A. octospinosus* and *A. echinator* colonies (Currie personal observation, more than 20 colonies).

My finding of lower abundance of actinomycete on minor workers as compared to major workers is unexpected, since these workers are specialized in tending the garden. Although minor workers typically have an abundance of the actinomycete on their laterocervical plates, it is not frequently found on other locations of their exoskeleton. Two possible explanations for this are: i) that the body size (i.e. surface area) of minor workers is not optimal for maintaining sufficient cultures of the bacterium to treat the garden for infection by

Escovopsis, and/or ii) that because of the garden-tending tasks performed by minor workers, presence of the actinomycete is only needed on the laterocervical plates. A third, but not mutually exclusive, possibility is that the actinomycete not only benefits the health of the garden, but protects the ants from entomopathogenic microbes. Although it appears that the actinomycete does not produce secondary metabolites with general anti-fungal properties that would help protect workers from ubiquitous entomopathogenic fungi (section 4.0), the possibility remains that specialized antibiotic activity is present that benefits the ants from an as yet unknown parasite(s) of the ants. In addition, the thick layer of filamentous bacterium (see figure 4.2a) would prevent any fungal spores from coming in contact with the exoskeleton of the ants, a common mode of entry for entomopathogenic fungi (Chamley 1984, St. Leger 1991); thus the bacterium may also serve as a physical barrier. If the actinomycete does help protect the ants themselves from microparasites, this may explain the pattern of abundance on different castes of *A. octospinosus*. Production of the large major workers is obviously more energetically costly to colonies than smaller minimums, and since younger workers have a longer life expectancy, they are more valuable to colonies because of the longer life-span remaining to serve the needs of the colony. Colonies may only receive a net energetic benefit from the heavy coverage of actinomycete when protecting the most valuable workers (e.g., young major workers). In fact, female reproductive alates (gynes) are energetically the most costly individuals for colonies to produce, and they typically have the highest abundance of actinomycete on their integument (see Table 5.1). Obviously, this heavy abundance of actinomycete is important for the establishment and maintenance of the vulnerable new fungal gardens that foundress queens attempt to establish. Future research on this tripartite mutualism should attempt to determine why different castes have different

amounts of actinomycete, as well as examine the potential role of the bacterium in protecting workers from entomopathogenic microbes.

The complexity of this ant-bacterial association is further illustrated by my findings that there is an increase in the abundance of the actinomycete on workers in response to infection of *Escovopsis*. My findings of increased abundance of the bacterium in *Escovopsis*-stressed colonies is further supported by the observation that both *Trachymyrmex* and *Acromyrmex* colonies stressed by sub-optimal gardening conditions and colonies experiencing an actual decline in garden volume tend to exhibit a much higher abundance of the bacterium on workers and/or the queen (Currie personal observation, Bot personal observation). This suggests that the ants utilize the actinomycetes as an inducible defense, with increased energy going toward growth of the bacterium when colonies are facing high parasite pressure, and less energy when parasite pressure is low. Inducible defenses are well documented in plants, and it is assumed that it is adaptive for the plant to avoid producing costly defenses in the absence of pathogens and/or herbivores (Karban and Baldwin 1997, Karban *et al.* 1999). In the fungus-growing ant mutualism, the actinomycete could be an energetically costly defense for colonies. In fact, in this study, there appears to be a slight increase in garden mass (although not statistically significant), in the absence of any *Escovopsis* infection, in those sub-colonies that have the bacterium removed (see Fig. 5.2). The source of nutrients supporting the growth of the fungus-growing ant-associated actinomycete is currently unknown. The filamentous bacterium does not appear to breakdown and consume the cuticle of the ants' exoskeleton. Extensive observations of older workers with the actinomycete removed using scanning electron microscopy revealed that no deterioration or penetration of

the ants' cuticle had occurred in the locations where the bacterium had been abundant (Currie unpublished data). One possibility is that the ants secrete nutrients for the growth of the bacterium at specialized locations of the integument, which could explain the genus-specific location of the bacterium, the age-specific abundance, and the apparent ability of the ants to promote the growth of the bacterium under stressed conditions.

Species can interact in harmful (parasitic), beneficial (mutualistic), and neutral (commensal) ways; but attempts to delineate symbiotic associations within these categories can be difficult (Herre *et al.* 1999). Therefore, determining the costs and benefits associated with interspecific interactions, especially mutualistic ones, is essential for understanding the stability and evolution of these associations (Cushman and Beattie 1991, Bronstein 1994, Herre and West 1997, Herre *et al.* 1999). My finding of a mutualistic bacterium protecting its symbiont from a pathogen might be a common but vastly overlooked benefit within mutualisms in general. Similar benefits have been suggested or shown in a few other mutualistic associations, including some mycorrhizae that suppress pathogens of their plant mutualists (Dehne 1982, Hooker *et al.* 1994, Kjølner and Rosendahl 1996) and some microbes associated with insects that produce antibiotics that may benefit their hosts (Dillon and Charnley 1986, Dillon and Charnley 1988, Boucias *et al.* 1996, Mueller in preparation). In addition, in humans, and likely many other animals, the normal bacterial flora of healthy intestinal and vaginal systems are thought to inhibit the establishment of harmful or pathogenic bacteria (Marrie *et al.* 1980, Cheng *et al.* 1995, Reid *et al.* 1995). Obviously, if a mutualist decreases the parasite pressure of its symbiont, this could result in a net benefit to itself. This may be especially important within mutualisms in which one partner has

undergone a transition from sexual to asexual reproduction (e.g. some lichenized algae, dinoflagellates symbiotic with marine invertebrates, and fungi associated with fungus-growing ants and termites). A decrease in sexual reproduction is theoretically predicted to result in an increase in parasite pressure because parasites are believed to be able to devastate genetically homogenous hosts. This theory, specifically referred to as the Red Queen hypothesis, suggests that parasites are a selective force maintaining sexual reproduction (Van Valen 1973, Jaenike 1978, Hamilton 1980). If, as proposed by Law and Lewis (1983), there is a selective advantage for one symbiont to be asexual, preventing it from escaping from its sexual host, then parasite pressure could be a significant cost to mutualism. This could select for mutualists to help protect their symbiont from parasites, or perhaps even result in establishment of additional mutualists within symbioses, as has occurred in the tripartite mutualism among attine ants, their cultivars, and their filamentous bacteria.

It is clear that clonal propagation of genetically homogenous cultivars for food by both ants and humans results in serious pathogen problems (Lucas 1980, Barrett 1981, section 2.0 and 3.0). Interestingly, attine ants, like humans, have resorted to chemical warfare with pathogens to maintain the health of their cultivars. It appears that both have failed to successfully develop a 'magic bullet' approach to disease and/or pest control. This is evident for human populations as illustrated by the extensive evolution of antibiotic and pesticide resistance in human diseases and agricultural pests. The lack of a 'magic bullet' in the attine ant symbiosis is illustrated by the finding that even though the actinomycete improves the ability of the ants to deal with *Escovopsis*, this pathogen still can cause dramatic damage in gardens (this study, section 2.0 and 3.0). In addition, despite the apparent selective pressure

of the actinomycete produced antibiotic on *Escovopsis*, the pathogen apparently has not evolved complete resistance over the long evolutionary history of this symbiosis. One possibility is that the bacterium and fungal parasite have been engaged in a co-evolutionary 'arms race' in which the bacterium evolves more potent metabolites to suppress *Escovopsis* and the parasite evolves increasingly greater resistance to the compounds. Identification of the metabolite(s) is now needed to gain a better understanding of the evolution of this fascinating tripartite mutualism. Increasing the understanding of the evolution of the secondary metabolites and the pathogen *Escovopsis* may provide important insight for medical practices and the evolution of antibiotic resistance in human pathogenic microbes.

Table 5.1. Abundance of actinomycete on minor workers, major workers, and gynes (incipient queens) in *A. octospinosus*. The proportion of individuals for each group was compared by assigning the amounts of actinomycete on each ant to one of two distinct groupings: none visible or just small coverage on the laterocervical plates (LP), or thick growth on the LP and present on other location on the body. Gynes were collected during a nuptial flight on May 9, 1997 in Gamboa, Panama.

Ant caste	None present or on the LP only	Thick covering of LP and other location on ants
Minor workers	80.3	19.7
Major workers	45.3	54.7
Gynes	4.2	95.8

Figure 5.1 Examination of the role of actinomycete in suppressing the growth of the parasite *Escovopsis* within *A. octospinosus* fungal gardens using a two-by-two factorial design crossing infection of the parasite with the presence/absence of the actinomycete on workers ($n = 5$ sub-colonies per treatment, mean \pm SE). a. Mean prevalence of the parasite *Escovopsis* within gardens ($X^2=30.24$, $df=1$, $P<0.0001$). b. Mean final garden mass of sub-colonies (infection treatment $F_{1,16}=0.0$, $P<0.99$; bacterial removal treatment $F_{1,16}=0.1587$, $P<0.6956$).

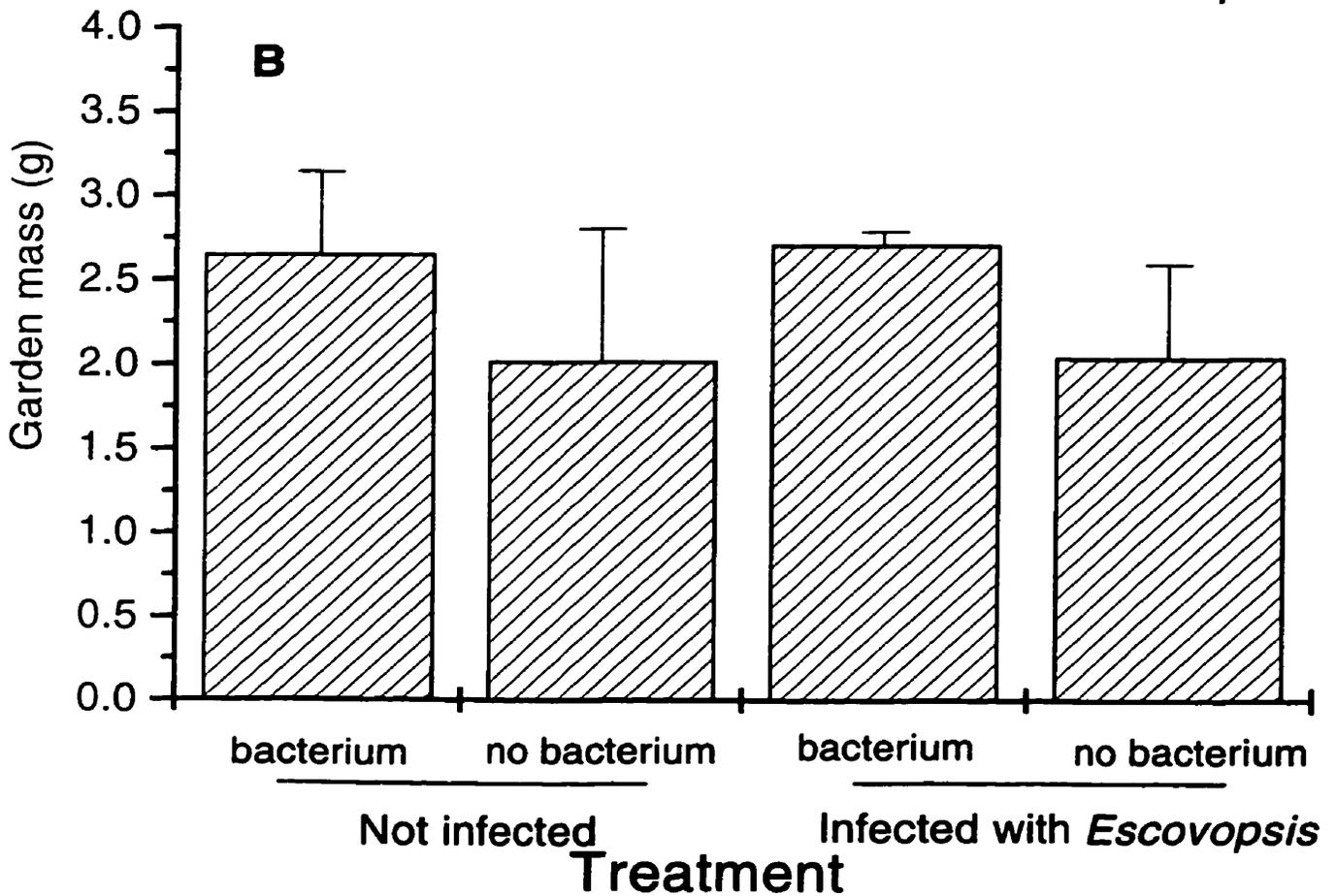
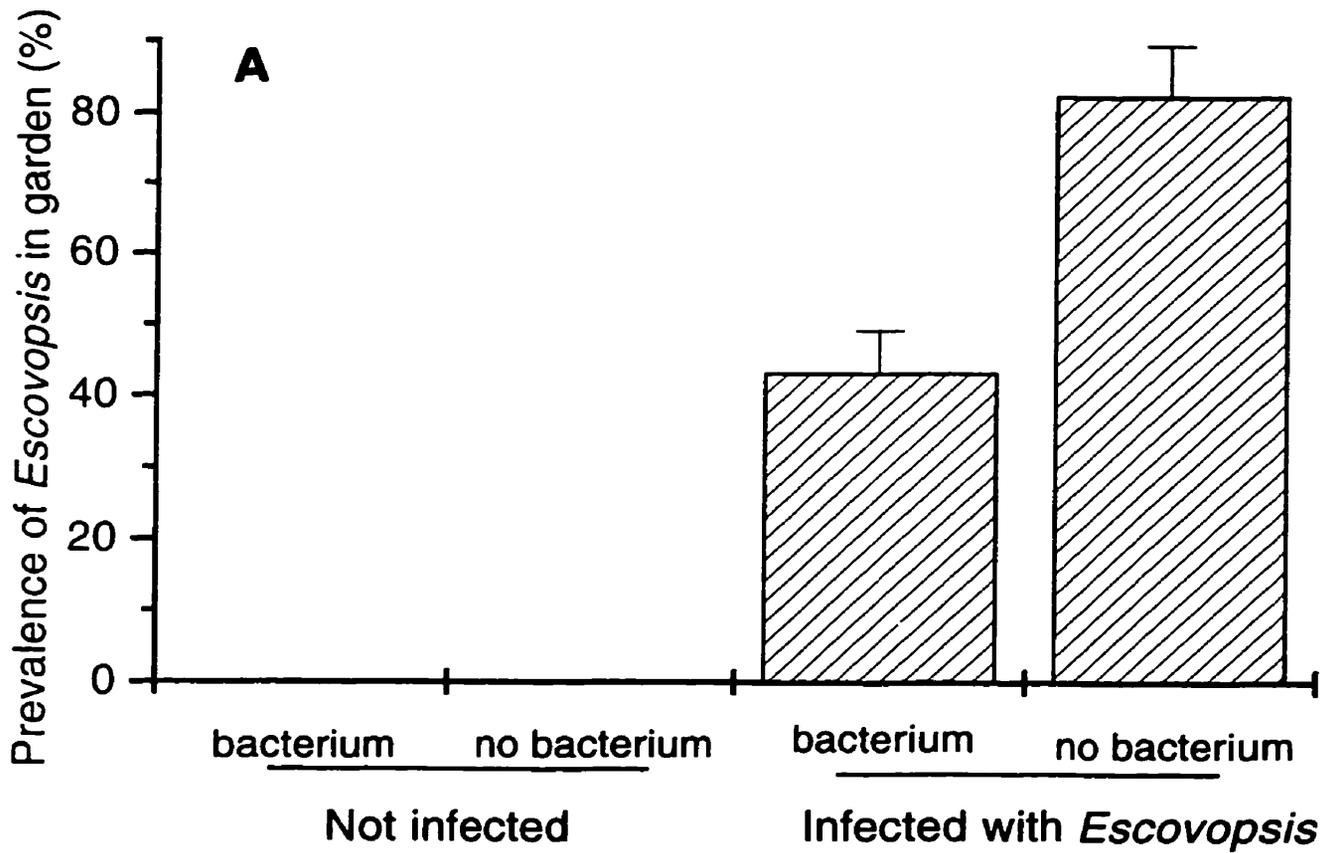


Figure 5.2 Mean final garden mass ($n = 36$, 12 sub-colonies from 3 different parent colonies, mean \pm SE) for the small sub-colonies used in the second experiment to examine the interaction between the ant-associated actinomycete and the garden parasite *Escovopsis* in a two-by-two factorial design cross experiment. Each sub-colony started with an initial mass of 80mg. An ANOVA reveals a significant interaction between the actinomycete and *Escovopsis* treatments ($F=5.72$, $df=1$, $P<0.0204$).

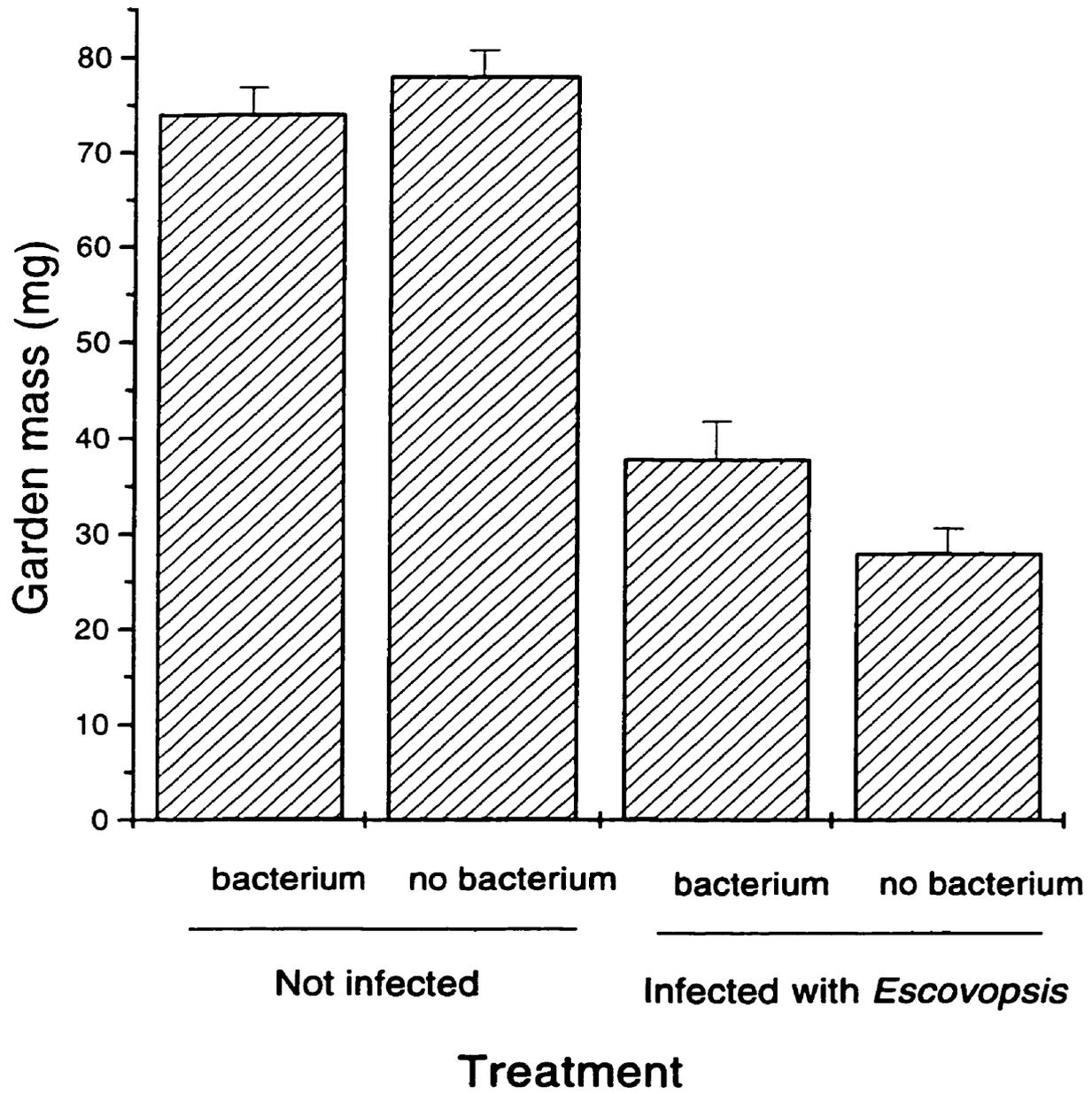


Figure 5.3 A comparison of the abundance of actinomycete on major workers tending the fungal garden versus foragers. Abundance of the bacterium on individual workers was characterized into two distinct groupings: none visible or present only on the laterocervical plates of the propleura (LP), or thick growth on the LP and other location on the body. A significantly higher abundance of bacterium is present on workers inside the garden as compared to foragers ($n = 3$, mean \pm SE, $X^2=82.00$, $df=1$, $P<0.0001$).

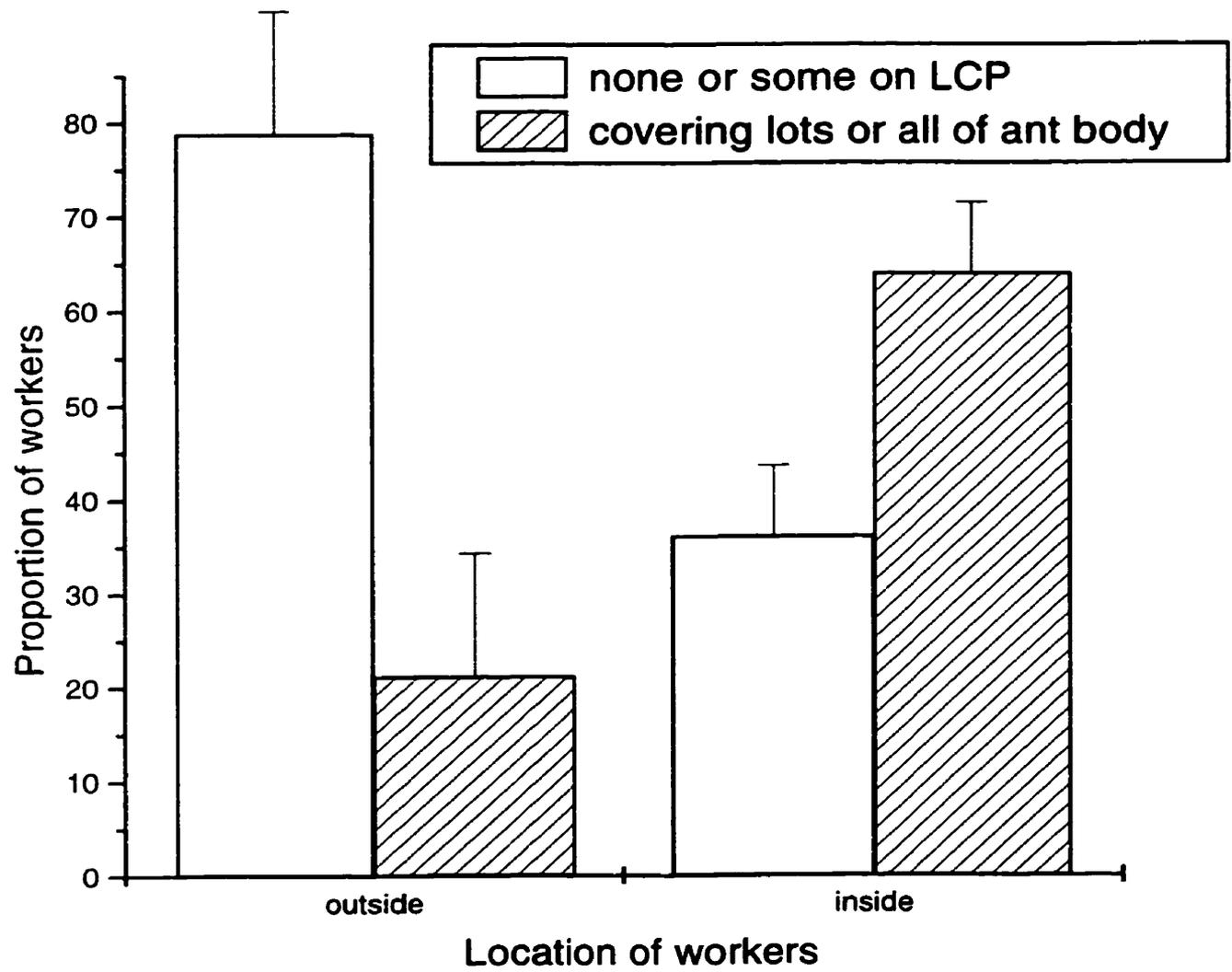
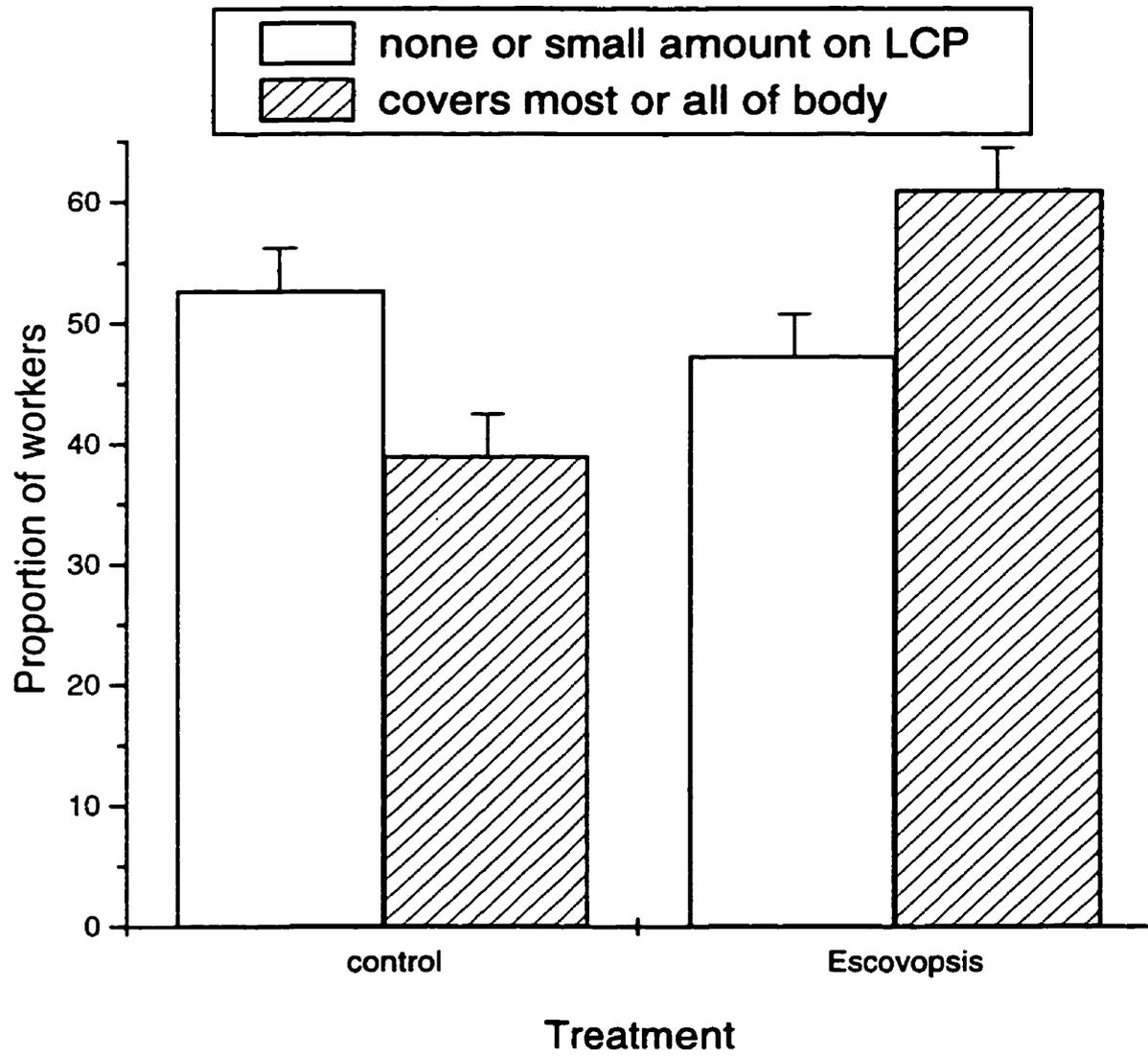


Figure 5.4 A comparison of the abundance of actinomycete on major workers from sub-colonies stressed with the parasite *Escovopsis* and unstressed colonies. The abundance of the bacterium on individual workers was placed into two distinct groupings: none visible or present only on the laterocervical plates of the propleura (LP), or thick coverage of the LP and other location on the body. A significantly higher abundance of bacterium is present on workers from *Escovopsis* infected colonies compared to uninfected colonies ($n = 3$, mean \pm SE, $X^2=8.24$, $df=1$, $P<0.0043$).



6.0 GENERAL DISCUSSION

The study of mutualism has traditionally lagged behind that of predation and competition, perhaps because of a common belief that mutualisms are generally not important (Boucher *et al.* 1982, Boucher 1985). With the recent realization that mutually beneficial interactions are of fundamental importance to all levels of biological organization, substantially more research attention has focused on such associations over the last few decades (section 1.0). However, Bronstein (1994) points out that our understanding of mutualism has been greatly hindered because most of the studies on beneficial interactions are primarily unilateral in approach, focusing on only one of two partners. This unilateral approach not only overlooks the presence of one partner, it also implies that mutualisms occur in isolation from all other organisms. In this thesis, I examine not only the bilateral interaction between two partners (fungus-growing ants and their fungi), but explore the interactions of these mutualists with other potential symbionts. Using this approach, I clearly establish the value of examining the diversity of interactions occurring within mutualisms in the broadest sense. In fact, I suggest that for an understanding of mutualisms, the interaction of these symbionts with other organisms is essential.

My research was conducted on the ancient and highly evolved association between fungus-growing ants and their fungi, which is a textbook example of mutualism and a model system in the study of symbiosis (Chapela *et al.* 1994, North *et al.* 1997, Mueller *et al.* 1998, Schultz 1999). In fact, research on this systems has resulted in the publication of thousands of scientific articles (Cherrett 1986, Mueller personal communication). However, despite the attention that this system has received by biologists, it is now obvious that the complexity of

this mutualism has been greatly underestimated. In the first part of my thesis, I establish that the ant fungal garden are parasitized by a virulent fungal parasite in the genus *Escovopsis*. This fungus has a dramatic impact on colonies by decreasing the rate of growth of the garden and workers, and has the potential to overwhelm the whole garden. In the second section of my thesis, I uncover another level of complexity in the mutualism between fungus-growing ants and their fungi; I establish the presence of a third mutualist within this symbiosis, a filamentous bacterium that produces antibiotics that suppress the growth of the specialized parasite *Escovopsis*. This bacterium occurs throughout the mutualism, from the phylogenetically basal to the phylogenetically derived genera (section 4.0 or Currie *et al.* 1999a). In fact, it appears that the ants have specialized structures for the maintenance of the bacterium, perhaps even glands that produce nutrients to support its growth (Currie unpublished data). These findings indicate that microbes and their metabolites may regulate the interactions of additional highly evolved symbioses.

6.1 MONOCULTURE

Traditionally, it has been assumed that fungus-growing ants grow their fungal gardens in axenic (pure) monocultures, thus excluding any microbial contaminants or parasites (Weber 1955, Weber 1966, Weber 1972, North *et al.* 1997). This is surprising for several reasons. First, organisms of low genetic diversity are theoretically predicted to be more susceptible to parasites (section 1.2.4). In addition, in the only well-studied analogous system, human agriculture, attempts to cultivate clonally propagated cultivars have led to serious difficulties with pests and pathogens. Parasites of social animals are also theoretically predicted to be more pronounced because the rate of parasite transmission typically increases due to the

close contact of group living (Alexander 1974, Freeland 1976, Brown and Brown 1986, Davies *et al.* 1991, Cote and Poulin 1995). Finally, some animal parasites are able to exploit the garden, highlighting its vulnerability. For example, species in the ant genus *Megalomyrmex* are parasites of this mutualism; they consume the fungus of the ants but do not help with its cultivation (Wheeler 1925, Weber 1940, Weber 1941, Brandão 1990). Without evidence to the contrary, it seems reasonable to assume that microbes, too, may threaten the ants' fungal gardens.

In this thesis, I establish that the fungal gardens are not maintained free of contaminants as previously assumed. The lower attines have ongoing problems with a diversity of common microbes (especially *Trichoderma* spp.), as demonstrated by the frequent isolations of common soil fungi from their gardens and occasional observations that these fungi overgrow gardens of lower attine ants (section 2.0 or Currie *et al.* 1999b, Currie unpublished data). This suggests that the lower attines are unable to maintain their gardens free of fungal contaminants, but instead must continuously contend with these alien microbes. In contrast, my research indicates that the higher attines have little difficulty with common fungi. I isolated few of these microbes from the gardens of higher attine ants, and the ants had little difficulty eliminating aggressive soil fungi (e.g., *Trichoderma*) that were sprayed onto their gardens (section 2.0, Currie in preparation). However, the gardens of higher attines have more serious and persistent infections of *Escovopsis*, thus establishing that the gardens of these ants are not maintained axenically. My research results establish that fungus-growing ant gardens are composed of a diversity of microbes, and that all the microbes in this association must be examined to fully understand the dynamics of this mutualism.

6.2 ESCOVOPSIS

Ironically, it was Möller (1893) who first suggested that fungus-growing ants maintain their gardens in 'monocultures', given that he also was the first to isolate and describe fungi that would later be described in the genus *Escovopsis*. In his 1893 monograph, he drew the conidiophores of a few species in this genus, and indicated that he observed these fungi overwhelming gardens of fungus-growing ants. However, Möller mistakenly assumed that what he was observing was the anamorph of the fungus cultivated by the ants, which was just growing out of control. At the time of his work, the concept of teleomorphs and anamorphs was still being developed, likely facilitating this mistake.

This fungal genus was later described as *Phialocladus* by Kreisel (1972), who isolated a strain from a nest of *Atta insularis* in Cuba. The name *Phialocladus* was later determined to be invalid by Muchovej and Della Lucia (1990), who renamed the genus *Escovopsis*. They named the type species *E. weberi* after the well-known ecologist, Neil Weber, who conducted extensive studies of the fungus-growing ants. Another species of *Escovopsis* was later isolated from a nest of *Trachymyrmex ruthae* (Seifert *et al.* 1995). This fungus produces globose phialide-bearing vesicles, thus having an appearance similar to that of the well-known genus *Aspergillus*, leading Seifert *et al.* (1995) to name it *E. aspergilloides*. These are the only two described species in this genus, however, this is a vast underestimate of the species diversity as I have obtained at least 8 to 10 new species (based on micromorphology) during my thesis work. In fact, it appears that there are actually several different genera.

In this thesis, I clearly establish that *Escovopsis* is a serious parasite of fungus-growing ants. In addition, I provide important insight into the host-parasite dynamics within this symbiosis. In section 3 of this thesis, I establish the spatial dynamics of the battle between the hosts and parasites. For example, the finding that *Escovopsis* is most prevalent in the older regions of gardens suggests that the parasite persists in this location, spreading upward into the fresher and nutrient-rich upper regions. The ants must continuously attempt to prevent the spread of this parasite to these newer locations. Another spatial scale of interactions occurs at the colony level. In colonies with multiple garden chambers, the mutualists must continuously attempt to prevent the spread of the parasite between chambers, while *Escovopsis* tries to establish further infections. Finally, at the population level, colonies of leaf-cutting ants are like islands of habitat for *Escovopsis*, thus the spread to new colonies is essential for its success. In contrast, attine ants would obviously benefit from preventing infections from becoming established. The host-parasite dynamics occurring within this quadripartite symbiosis are very complex and warrant further study.

Obviously we are just beginning to understand the dynamics of *Escovopsis*, with many basic and important questions remaining to be addressed. However, there are three questions that are of particular importance and theoretical interest. First, what is the teleomorphic (sexual) stage of *Escovopsis*, or does it occur only as an anamorph? My attempts to promote the fruiting of *Escovopsis* in culture and within gardens have been unsuccessful (Currie unpublished data). In addition, I failed to discover it during my numerous observations of fungus-growing ants in the field. It is possible that *Escovopsis* does not have a teleomorphic stage, however much more work needs to be done in this area. Some promising locations to

search include the dump of the ants and colonies that are completely over-grown by the parasite in the field.

The second question outstanding is, what is the evolutionary origin of *Escovopsis* within this quadripartite symbiosis? I propose three likely scenarios for the origin of this parasite. First, it is possible that the ancestor of *Escovopsis* is a parasite of the free-living relatives of the fungi that the ants cultivate. Although my attempts to isolate *Escovopsis* from the closely related relatives of the ants' cultivars were unsuccessful, this is still a very promising hypothesis. Second, it is possible that *Escovopsis* evolved from being a parasite of the ants themselves, switching hosts at some point over the evolutionary history of this association. No *Escovopsis*-like parasites of ants were found during this study, but this is still a possibility. Finally, it is possible that the ancestor of *Escovopsis* occurs in the substrate that the ants use to manure their fungal gardens. To address this question the evolutionary history of *Escovopsis* should be reconstructed so the life history of fungi that are closely related to this genus can be examined.

The third question that needs to be addressed, now that it is clear that the parasite is not transmitted vertically by queens during their nuptial flight, is how is *Escovopsis* dispersed between colonies? *Escovopsis* is a wet-spored fungus, so it is obviously not air-dispersed, suggesting that it is likely vectored between colonies. It is difficult to envision how to even begin to identify the possible vectors that might be involved. The most promising candidates are the diverse assemblage of invertebrates that live in close association with fungus-growing ants, and that occasionally move between colonies, potentially carrying *Escovopsis* with

them. An additional possibility is that *Escovopsis* has another life history stage that is involved in dispersal. For example, its teleomorph could occur on another host, which facilitates the spread of *Escovopsis* to new colonies. An understanding of the dispersal of *Escovopsis* is an important life history trait that, if established, should provide more insight into the ecology and evolution of fungus-growing ants.

6.3 BIOLOGICAL CONTROL

Leaf-cutting ants are not only important ecologically (see section 1.3.1), they also are a major pest in forestry and agriculture in the new world tropics, doing over a billion dollars in damage (Hölldobler and Wilson 1990). Current methods for controlling leaf-cutting ants are ineffective. In addition, the unauthorized use of very toxic pesticides and even motor oil is a common method that is currently employed by many neotropical farmers to attempt to eliminate these ants. My discovery that leaf-cutting ant fungal gardens are host to a virulent parasite provides new promise for developing effective and safe methods for controlling leaf-cutting ants in agricultural areas. However, any attempt to utilize *Escovopsis* for the biological control of leaf-cutting ants must be done with extreme caution. Leaf-cutting ants are an important component of the natural functioning of tropical rainforests, and as such, their presence in these ecosystems should not be compromised. Therefore, attempts to introduce new or genetically engineered strains of the parasite into leaf-cutting ant populations should not be undertaken. Instead, I encourage biological control that augments local strains of *Escovopsis* within populations of leaf-cutting ants, increasing the impact of the parasite without the possibility of creating an epidemic that could potentially wipe out leaf-cutting ants in regions of tropical rainforest. In any case, a better understanding of both

the dynamics of the interactions between this parasite and the ant-fungus-bacterium mutualism and the mechanisms of dispersal is needed before any biological control measures using *Escovopsis* should be considered.

6.4 RED QUEEN AND THE CO-EVOLUTIONARY ‘ARMS RACE’

My examination of parasites of the fungal gardens of attine ants provides empirical support for the Red Queen hypothesis. As noted above, the fungal gardens of the more derived genera of attine ants are ancient asexual clones, while the phylogenetically basal genera cultivate fungi that are frequently re-acquired from free-living, sexually reproducing fungi. As predicted by the Red Queen hypothesis, the long evolutionary history of clonal propagation in the higher attines has resulted in more serious parasite pressure. In this thesis, I show that *Escovopsis* is much more prevalent in the gardens of the higher attines than in the lower attines. In addition, infecting colonies of the higher attines with *Escovopsis* results in greater garden loss and higher mortality as compared to infecting colonies of the lower attines (Currie unpublished data). Further work experimentally examining the virulence of *Escovopsis* within this mutualism would be valuable.

The interactions between fungus-growing ants and *Escovopsis* appears to be ancient. *Escovopsis* occurs throughout the geographic range of the mutualism, having been isolated from regions spanning Texas and Brazil (section 2.0). In addition, this parasite occurs across the phylogeny of the ants, from the phylogenetically basal genera (i.e., *Myrmicocrypta*, *Mycocepurus*, and *Apterostigma*) to the phylogenetically derived leaf-cutting ants (i.e., *Acromyrmex* and *Atta*). Based on both morphological and molecular evidence, the species of

Escovopsis that occur in the basal genera are a distinct clade from the isolates that occur in the higher attines (Currie unpublished data). Thus it is possible that the attine symbiosis is a co-evolutionary ‘arms race’ between *Escovopsis* and the tripartite association among the actinomycete, the ants, and the fungal cultivars. In fact, it is possible that this arms race has been occurring for millions of years. Studies examining the evolutionary history of *Escovopsis* and the ant-associated actinomycete should provide fascinating new insight into the dynamics of this mutualism, as well as into the process of co-evolution in general.

6.5 PARASITES OF MUTUALISMS

As noted above, mutualisms are generally considered to occur in isolation from other organisms, free of parasites. One major exception to this is the presence of ‘cheaters’: taxa that are closely related to one of the mutualists and have evolved an ability to exploit the other symbiont by obtaining a reward without reciprocating a benefit (section 1.0). The presence of the specialized and virulent parasite *Escovopsis* within fungus-growing ant colonies suggests that highly evolved parasites might be present in other mutualistic associations. This may be especially true of mutualisms involving symbionts of low genetic diversity, such as those involving one symbiont that reproduces asexually. Future work should look for unrelated parasites in other highly evolved mutualisms.

6.6 RELEVANCE FOR HUMAN SURVIVAL

As indicated at the beginning of this thesis, an understanding of symbiosis is not only important for understanding the world and our place within it, it is also increasingly apparent that it is essential for human welfare (section 1.1). My research not only provides important

insight into the dynamics of symbiosis, it also has implications for human survival (Schultz 1999, Wilkinson 1999). The most obvious example of this is in relation to the evolution of resistance to antibiotics by human pathogens. Microbial pathogens of humans have developed resistant to our antibiotics rapidly over the 60 year history of antibiotics use by humans. In contrast, fungus-growing ants have been successfully using actinomycetes for the production of antibiotics for more than 50 millions years. Studies examining how these ants have been so successful at using antibiotics for so long could provide important insight into our own use of these incredibly important pharmaceuticals.

My research also has important implications for the development of new antibiotics. Current searches for promising new antibiotics to treat human diseases are mostly done by conducting massive screenings of a random collection of organisms. However, it has recently been argued that it would be better to use a biologically informed approach to this search (Eisner 1990, Beattie 1992). The actinomycete associated with attine ants produces secondary metabolites that are specific toward *Escovopsis*, indicating that these microbes likely have little promise for producing pharmaceutically useful compounds. However, my discovery suggests that other highly evolved mutualisms could be ideal systems for searching for promising antibiotics.

6.7 CONCLUSIONS

One reason that humans are fascinated with fungus-growing ants is the interesting parallel between the two organisms based on the fact that both practice agriculture (Hölldobler and Wilson 1990, Mueller *et al.* 1998, Diamond 1999). However, agriculture in fungus-growing

ants predates the origin of human agriculture by more than 50 million years (Wilson 1971, Mueller *et al.* submitted). My thesis draws two new parallels between humans and fungus-growing ants and their fungi. First, like humans, these ants face serious problems with diseases of their cultivars, especially when the latter are clonally propagated. In addition, fungus-growing ants have established, through the process of natural selection, an association with bacteria for the production of antibiotics. Humans view the discovery of antibiotics by Alexander Fleming over 60 years ago as one of the major achievements in the history of medicine. Again, ants beat us to it, and are apparently beating us at it.

7.0 GENERAL SUMMARY

The ancient and highly evolved mutualism between fungus-growing ants (Formicidae: Attini) and their fungi (Agaricales: mostly Lepiotaceae) is a textbook example of symbiosis. The ants carefully tend the fungus, which serves as their main food source, and traditionally are believed to be so successful at fungal cultivation that they are able to maintain the fungus free of microbial parasites. This assumption is surprising in light of theories on the evolution of parasitism, especially for those species of ants that have been clonally propagating their cultivars for millions of years.

In this thesis, I conduct the first extensive examination of parasites attacking the fungus gardens of attine ants. I establish that the gardens are host to specialized parasitic fungi in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales). I isolated *Escovopsis* from the gardens of ants representing the generic diversity of Attini, suggesting that this is an ancient parasite of ant fungal gardens. Contrary to the traditionally held assumption that parasites with a long evolutionary history with their host evolve towards being benign, *Escovopsis* is a very virulent parasite of this mutualism which may devastate gardens rapidly. In addition, persistent infections of *Escovopsis* result in major decreases in the growth rate of infected colonies, both in terms of fungal biomass and numbers of workers. I also establish that *Escovopsis* is horizontally transmitted between colonies, as would be predicted based on the virulence of this parasite.

The mutualism between fungus-growing ants and their fungi appears to be a good system for testing the Red Queen hypothesis, a theory predicting that the adaptation by parasites to

genetically homogenous hosts is a selective force maintaining sexual reproduction. The fungal cultivars of the derived genera of fungus-growing ants are asexual clones of ancient origin, whereas the phylogenetically basal lineages of attine ants cultivate recently acquired cultivars from sexually reproducing free-living stocks. As predicted by the Red Queen hypothesis, the ancient clones of the phylogenetically derived genera appear to have more severe parasite pressure from *Escovopsis* than those from the phylogenetically basal ant species.

The gardens of fungus-growing ants are continuously inoculated with competitively superior microbes present in the substrate the ants use to manure their fungal cultivars. Successful fungiculture is also threatened by the virulent, specialized parasite *Escovopsis*. As the ants and their fungi are mutually dependent, the maintenance of stable fungal monocultures in the presence of weeds or parasites is critical to the survival of both organisms. Nonetheless, current explanations of how stable fungicultures are maintained are lacking.

In the second section of this thesis, I establish a completely new and fundamental mechanism employed by the ants to maintain the health of their fungal cultivars: a mutualistic association with actinomycetes (a group of filamentous bacteria that are well-known for their ability to produce antibiotics). The actinomycete is present in all species of fungus-growing ants examined and is carried upon regions of the ants' cuticle that appear to be specialized. Furthermore, the location of the bacterium is genus specific, occurring under the forelegs in the phylogenetically basal genera, and on the laterocervical plates of the propleura in the more phylogenetically derived genera. This suggests that the association between the

bacterium and fungus-growing ants is ancient. I also found the bacterium to be present on queens during their mating flight, indicating that it is primarily vertically transmitted between colonies. Bioassays failed to detect the production of any general antifungal metabolites by the ant associated bacterium, but revealed potent metabolites that target *Escovopsis*, the specialized parasites of the ants gardens. In addition, at least in the lower attines, the actinomycete produces compounds that stimulate the growth of the fungal mutualist.

My findings clearly establish that fungus-growing ants have an ancient and highly evolved symbiosis with actinomycetes. In addition, the discovery that the secondary metabolites inhibit *Escovopsis* suggests that this association is mutualistic, with the bacterium helping the ants protect the garden against the virulent parasite and the ants providing nutrients for the bacterium to grow. The production of secondary metabolites is energetically costly and requires complex biosynthetic pathways, so the maintenance of metabolites specific towards *Escovopsis* presumes a benefit to the actinomycete and thus is strong evidence of this association being mutualistic. Nevertheless, considering the implications of this discovery for our understanding of the mutualism between attine ants and their fungi, and of symbiosis in general, it is important that the benefit of the bacterium for suppressing *Escovopsis* be demonstrated within colonies. I conducted a two-by-two factorial design experiment, crossing the presence/absence of actinomycete with the presence/absence of *Escovopsis* to establish the role of the bacterium in suppressing the parasite within sub-colonies of fungus-growing ants. Using this design, I established that sub-colonies with the bacterium present were significantly more able to deal with infections of *Escovopsis* than those removed. In addition, as would be predicted if this bacterium is important for the maintenance of the

health of the garden, garden tending workers had a higher abundance of the bacterium than foraging individuals.

In this thesis, I establish that the textbook mutualism between fungus-growing ants and their fungi is actually a quadripartite symbiosis. In fact, my findings suggest that the attine symbiosis is a co-evolutionary 'arms race' between the garden parasite *Escovopsis*, on the one hand, and the tripartite association amongst the actinomycete, the ants, and the fungal mutualist on the other. These findings completely change our understanding of this textbook association, but more importantly, have far-reaching implications for symbioses in general. The importance of both *Escovopsis* and the actinomycete in this mutualism suggests that microbes may mediate the interactions occurring in other mutualisms. In addition, it suggests that studying the highly evolved chemical interactions occurring between *Escovopsis* and the fungus-growing ant associated actinomycetes may provide valuable theoretical and practical insights into the discovery and use of antibiotics.

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