ABSTRACT

YOUNGSTEADT, ELSA KRISTEN. Neotropical Ant-Gardens: Behavioral and Chemical Ecology of an Obligate Ant-Plant Mutualism. (Under the direction of Coby Schal.)

Seed dispersal mutualisms are essential for the survival of diverse plant species and communities worldwide. An outstanding but poorly understood ant-seed mutualism occurs in the Amazonian rainforest, where arboreal ants collect seeds of several taxonomically diverse plant species and cultivate them in nutrient-rich nests, forming abundant hanging gardens known as ant-gardens (AGs). AG ants and plants are dominant members of lowland Amazonian ecosystems, and their interaction is obligate and apparently species-specific. Though established AGs are limited to specific participants, it is unknown at what stage specificity arises. Seed fate pathways in AG epiphytes are undocumented, and the recognition cues that mediate the mutualism are unknown. Here the species specificity of the AG ant-seed interaction is assessed, and chemical cues are characterized that elicit seed-finding and seed-carrying in AG ants.

To examine the specificity of the ant-seed interaction, general food baits and seeds of the AG plant *Peperomia macrostachya* were offered on alternate days at 108 bait stations. Seventy ant species were detected at food baits and could have interacted with AG seeds, but only three species collected *P. macrostachya* seeds, and 84% of observed seed removal by ants was attributed to *C. femoratus*. In a separate experiment, arthropod exclusion significantly reduced AG seed removal rates, but vertebrate exclusion did not. Thus species specific seed dispersal, rather than post-dispersal processes, appears to be the primary
determinant of the distribution of AG plants. The results also begin to quantify factors that affect seed fate in this unusually specific plant-animal mutualism.

To characterize behavioral cues on AG seeds, solvent extracts of three species (Anthurium gracile, Codonanthe uleana, and P. macrostachya) were subjected to chromatographic fractionation. To test behavioral activity, each fraction was applied to other seeds that ants ordinarily ignore. At least one fraction of each seed extract elicited retrieval behavior in C. femoratus, but the active fractions of the three species differed in chemical composition, indicating that each plant species elicits seed-carrying with a different class of chemical attractants.

Volatile attractants from P. macrostachya were further investigated using Y-tube olfactometer assays and gas chromatography-electroantennographic detection. In the olfactometer, C. femoratus preferred the odor of P. macrostachya seeds over that of control seeds, and P. macrostachya extract over solvent control. Five electrophysiologically active phenolic and terpenoid compounds from P. macrostachya extract were identified and combined in a synthetic blend, which was also preferred over solvent control and over a single component alone. Although seed dispersal by ants is common worldwide, this is the first documentation of volatile attractants from any ant-dispersed seed. Furthermore, the most abundant component of the attractive blend, geranyl linalool, is toxic to many ant species, hinting at one mechanism by which AG seeds may avoid being collected by inappropriate dispersers.
Future studies of AGs should address costs and benefits of the interaction to its various participants, evaluate the consequences of the interaction for gene flow and population structure in the plants, and assess geographic variation in ant and seed traits that facilitate the interaction. Such studies would bear on central themes in the theory of mutualism, including the origin of mutualism, the evolution and coevolution of traits that facilitate the interaction, evolution and maintenance of species-specificity, and prevention of cheating. Furthermore, the taxonomic diversity of AG participants provides a unique opportunity to examine these questions in distantly related species that have all converged upon a similar mutualistic survival strategy.
Neotropical Ant-Gardens: Behavioral and Chemical Ecology of an Obligate Ant-Plant Mutualism

by
Elsa Kristen Youngsteadt

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Entomology

Raleigh, North Carolina

2008

APPROVED BY:

____________________________   ____________________________
Fred Gould   Jules Silverman

____________________________   ____________________________
Edward L. Vargo   Coby Schal
Chair of Advisory Committee
Elsa Youngsteadt was born in Yellville, Arkansas, on February 3, 1979, and grew up under the direction of her parents Norman and Jean Youngsteadt in Springfield, Missouri. There she was captivated at a young age by the distant tropics and, as a fifth grader, aspired to study primate behavior in Madagascar. Years later, researching ant behavior in Perú fit rather well with her life plans, and satisfied her adventurous and romantic notions of tropical biology. Nevertheless, finding herself with a lesser aptitude—or perhaps appetite—for lab work, she began crafting an alternative future in science writing. Beginning with Technician, NC State's student newspaper, in 2005, one thing led to another and eventually she spent ten weeks reporting science stories on the air for WOSU Public Radio in Columbus, Ohio. Shortly thereafter, she spent another six months writing news for Science magazine in Washington, DC. There she lived in a miserable bedbug-infested house, but did run her first marathon and enjoyed several idyllic climbing trips in West Virginia. Although she hopes to make herself useful at low latitudes again someday, her immediate plans include working as a science reporter or editor, nudging the sustainable agriculture revolution along its way before it's too late, and expanding her menagerie, which presently comprises only one patient boa constrictor named Chantico. Elsa's various other legitimate activities are documented in the vita below.
EDUCATION

M.S., Biological Sciences, University of Wisconsin-Milwaukee, 2003
Thesis: The effects of ants on the carnivorous butterfly caterpillar *Feniseca tarquinius* and the role of chemical camouflage in the *Feniseca*-ant interaction.
B.A., Biology, Truman State University, Kirksville, Missouri, *summa cum laude*, 1999

SCIENCE WRITING EXPERIENCE

- Reported and wrote 25 online news articles for *ScienceNOW*, 5 print news and feature stories for *Science*, and about 30 short news items.
- Researched and recorded portions of 5 *Science* podcasts.
- Collaborated with the Art and News departments to develop an interactive online infographic.

AAAS Mass Media Fellow, WOSU Public Media, Columbus, OH, June-August, 2007
- Prepared 20 news and feature stories for public radio during nine-week fellowship.
- Found leads, researched, interviewed, collected sounds, wrote and voiced scripts, posted articles and audio files online.

Science Writing Intern, National Evolutionary Synthesis Center, Durham, NC, January 2007-January, 2008
- Created online resource for biology educators by writing 12 "Evolution in the News" articles to summarize and explain current research in evolution, relate it to textbook principles, and provide questions for classroom discussion.

Freelance Writer, July, 2006-present

Staff Writer, *Technician*, daily student newspaper of North Carolina State University, July, 2005-September, 2006
- Contributed more than 30 articles to the Science and Tech and News sections, generated story and graphic ideas and brainstormed at staff meetings.

OTHER PROFESSIONAL EXPERIENCE

Research and Teaching Assistant, North Carolina State University Structural Pest Management Training & Research Center, Raleigh, NC, July, 2008-present
- Assist with training programs on basic biology and identification of urban pests.

Teaching Assistant, University of Wisconsin-Milwaukee, Milwaukee, WI, August, 2000-May, 2001
- Taught laboratory sections of zoology class for non-majors and tutored students.
• Monitored populations of endangered butterflies and moths and recommended habitat management strategies.

Curator, Collection Manager, Educator, and Research Assistant, Milwaukee Public Museum, July, 1999-October, 2002
• Managed research collection of Lepidoptera.
• Maintained and managed butterfly vivarium.
• Educated museum guests about butterfly biology.
• Collected field data for a sustainable agriculture research program in Costa Rica.

Assistant Supervisor, World Bird Sanctuary Birds of Prey Show, Milwaukee County Zoo, May-August, 1997; May-July, 1999
• Coordinated team of 5 to produce daily bird shows and maintain and train 20 birds of prey. Presented one to three shows per day, for audiences of up to 100.
• Promoted from educator/bird trainer in 1997 to assistant supervisor in 1999.

AWARDS AND FELLOWSHIPS

2008 North Carolina Entomological Society Outstanding Ph.D. Student Award
2007 First place poster in behavior and ecology, Entomological Society of America annual meeting
2007 AAAS Mass Media Fellowship
2006 First place, Keck Center for Behavioral Biology essay contest
2006 GAANN Biotechnology Fellowship
2005 Amazon Conservation Association Research Fellowship
2004 North Carolina Entomological Society Travel Award
2004 Sigma Xi Grant in Aid of Research
2000 University of Wisconsin-Milwaukee Graduate School Fellowship
2000 National Science Foundation Predoctoral Fellowship
1995 National Merit Scholarship
1995 Truman State University Pershing Scholarship (full ride)

PEER-REVIEWED PUBLICATIONS


OUTREACH AND COMMUNITY SERVICE


Kucinich for President campaign Raleigh coordinator, Raleigh, NC, 2003-2004. Created support network, conducted meetings, organized canvassing events.


Center for the Spanish Speaking English tutor, Milwaukee, WI, 2000-2002. Guided adult ESL students in English exercises to prepare for citizenship and driver license tests.

Jardin Gaia Wildlife Rescue Center volunteer, Quepos, Costa Rica, 1998. Treated and maintained injured wildlife for release, created educational collection of local butterflies, developed butterfly vivarium, led tours of the center.

ADVENTURES

Rock Climbing, traditional lead-climbing, 2008-present

Long distance running, marathons/half-marathons, 2007-present

Motorcycles, riding/touring, 2007-present

Sustainable agriculture, volunteer farm work, 2006-2008

Raleigh Civic Symphony, viola section, 2003-2005

LANGUAGES

English (fluent)

Spanish (fluent)

French (intermediate)
# TABLE OF CONTENTS

LIST OF TABLES .......................................................................................................................... vii
LIST OF FIGURES ......................................................................................................................... viii
ANT-GARDENS: BEHAVIOR, ECOLOGY, AND EVOLUTION IN A UNIQUE ANT-PLANT MUTUALISM ............................................................... 1
  Ant-gardens: Definition and ecological role ................................................................. 4
  Ant-gardens as seed dispersal mutualism ................................................................. 7
  Ant-gardens as ant-plant symbioses ....................................................................... 17
  Ant-gardens as parabioses ..................................................................................... 22
  Evolution of the AG symbiosis ............................................................................ 27
  Conclusion .............................................................................................................. 31
  Literature cited ....................................................................................................... 33
SPECIES-SPECIFIC SEED DISPERSAL IN AN OBLIGATE ANT-PLANT MUTUALISM .......................................................... 51
  Introduction .............................................................................................................. 53
  Results .................................................................................................................... 56
  Discussion ............................................................................................................... 59
  Materials and Methods ......................................................................................... 68
  References ................................................................................................................ 73
SEED ODOR MEDIATES AN OBLIGATE ANT-PLANT MUTUALISM IN AMAZONIAN RAINFORESTS .................................................. 89
  Results ..................................................................................................................... 93
  Discussion ............................................................................................................... 96
  Materials and Methods ......................................................................................... 101
DIVERGENT CHEMICAL CUES ELICIT SEED COLLECTING BY ANTS IN AN OBLIGATE MULTI-SPECIES MUTUALISM IN LOWLAND AMAZONIA .................................................................................................................. 115
  Methods .................................................................................................................... 120
  Results ..................................................................................................................... 127
  Discussion ............................................................................................................... 131
  Literature cited ....................................................................................................... 138
LIST OF TABLES

CHAPTER 1

Table 1. Ant species that construct AGs, and epiphyte species that produce seeds dispersed by these ants, that are restricted to AGs, and/or are frequently reported in AGs ......................................... 45

Table 2. Occurrence of nine volatile compounds in AG seeds ............................ 47

Table 3. Summary of characteristics of parabiotic ants ....................................... 48

CHAPTER 2

Table 1. Ant species recorded at general food baits and at ant-garden seed baits on transects in southeast Perú ............................................... 80

Table 2. Results of ANOVA testing for fixed effects of cage, Tanglefoot and AG ants in the exclusion experiment .............................................. 82

CHAPTER 4

Table 1. Occurrence of seven volatile compounds in AG and non-AG epiphytes ................................................................................................ 144

Table 2. Results of ANOVA on ranks, testing for effects of extract type (hexane or methanol extracts of P. macrostachya, C. uleana, or blanks) on the order in which ants retrieved test seeds ......................... 146

Table 3. Ant response to crude extracts of AG seeds prepared with different solvents and presented in seed carrying assays ......................... 147

Table 4. Sugar and amino acid content of methanol fractions of A. gracile and C. uleana extracts ............................................................... 148
LIST OF FIGURES

CHAPTER 1

Figure 1. Structures of compounds found in AG seeds (see Table 1) ................. 50

CHAPTER 2

Figure 1. Ant garden in southeast Perú ................................................................. 83
Figure 2. Fate of *P. macrostachya* seeds placed at 108 sampling stations ........... 84
Figure 3. Arthropod and vertebrate exclusion experiments ............................... 85
Figure 4. Removal of *P. macrostachya* seeds during exclusion experiments ...... 86
Figure 5. Actual and putative seed fate pathways for *P. macrostachya* seeds ..... 87

CHAPTER 3

Fig. 1. Arboreal ant-garden involving the ants *Camponotus femoratus* and *Crematogaster* cf. *limata parabiotica* and the epiphytic plants *Anthurium gracile* and *Peperomia macrostachya* ....................... 109
Fig. 2. Response of *C. femoratus* ants to different doses of *P. macrostachya* extract in seed-carrying assays ......................................................... 110
Fig. 3. Behavioral tests of olfactory attractants from AG seeds ....................... 111
Fig. 4. Representative GC-EAD trace of *Camponotus* antenna response to a 0.25 seed equivalent of the behaviorally active fraction of *P. macrostachya* extract, separated on a nonpolar column ........................ 113
Fig. 5. Behavioral confirmation that a blend of five electrophysiologically active compounds from ant-garden seeds attracts *C. femoratus* ants ............................................................. 114

CHAPTER 4

Figure 1. Structures of compounds frequently detected in AG seeds, but not in non-AG congeners (see Table 1) ............................................................. 149
Figure 2. AG ants (*C. femoratus*) preferred hexane extracts of *P. macrostachya* and methanol extracts of *C. uleana* in the seed-carrying assay ................................................................. 150
Figure 3. Ants preferred different chromatographic fractions of *A. gracile*, *C. uleana* and *P. macrostachya* extracts in the seed carrying assay ............................................................. 151
Chapter 1

Ant-Gardens: Behavior, Ecology, and Evolution in a Unique Ant-Plant Mutualism

This chapter was prepared for submission to Biotropica with coauthor Coby Schal.
ABSTRACT.

Ant-plant interactions, particularly ant-plant protection mutualisms and seed-dispersal by ants, have contributed much to current understanding of the evolution and ecology of mutualism. A third form of ant-plant mutualism, the ant-gardens (AGs), has so far lagged behind in its contribution to this body of knowledge but may be ripening as a further source of insight. AGs are formed when ants collect seeds of specific plants and embed them in arboreal carton nests. Participants in this obligate mutualism are taxonomically diverse, abundant and ecologically important, thus providing the opportunity to examine how multiple unrelated species have converged on what appears to be the same mutualistic survival strategy. Recent work has begun to clarify benefits of the AG interaction to participating species, examine the chemical basis of the ant-seed interaction, and demonstrate the species-specificity of the ant-seed interaction. Missing are long-term studies of the costs and benefits of the interaction to its various participants, evaluation of the consequences of the interaction for gene flow and population structure in the plants, and studies of geographic variation in ant and seed traits that facilitate the interaction. Such studies would bear on central themes in the theory of mutualism, including the origin of mutualism, the evolution and coevolution of traits that facilitate the interaction, evolution and maintenance of species-specificity, and prevention of cheating.
MUTUALISM—cooperation among species—is a ubiquitous force in the origin and maintenance of earth's biological complexity, influencing all levels of organization from single cells to ecosystems (Janzen 1985, Maynard Smith 1989, Margulis 1991, Currie et al. 2003, Bronstein et al. 2006). As a relative latecomer to ecological theory, mutualism has received serious attention only in recent decades, and a more complete understanding of mutualism still depends upon clarifying the origin, costs, and benefits of specific interactions, the variability of these interactions in different ecological contexts, and the factors that promote or prevent species specificity and cheating (Bronstein 1994, Herre et al. 1999, Currie et al. 2003, Stanton 2003, Sachs et al. 2004, Douglas 2008, Stadler & Dixon 2008). In the past twenty years, ant-plant interactions have emerged as an important testing ground and source of insight into the evolution and ecological role of mutualism (Davidson & McKey 1993, Bronstein 1998, Heil & McKey 2003, Bronstein et al. 2006, Guimarães et al. 2006). Protection mutualisms—in which plants reward ants with food and/or housing in return for defense against herbivores and/or competitors—have figured most prominently in this literature, with good reason: they have evolved repeatedly in hundreds of ant and plant genera, and range from general facultative interactions to specific obligate symbioses, providing ample opportunity for comparative and manipulative studies (Yu & Davidson 1997, Bronstein 1998, Heil & McKey 2003, Webber et al. 2007). A second outstanding ant-plant mutualism is myrmecochory, or seed dispersal by ants. This often diffuse interaction has also arisen many times and is integral to ecosystem regeneration in several regions of the globe (Beattie & Hughes 2002, Bronstein et al. 2006). In a third category of ant-plant
mutualism, the ant-gardens (AGs), ants collect seeds of epiphytic plants and cultivate them in arboreal carton nests. This interaction combines elements of both seed-dispersal and protection mutualisms, as well as nutritional exchange among participants and, often, the cohabitation of two or more ant species in the same nest-garden and close association with hemipterans. The AG interaction is obligate and restricted to a relative handful of participating species, but has evolved independently in several plant and ant taxa. Such intricacies make AGs the most complex ant-plant mutualism known (Hölldobler & Wilson 1990). Since AGs were first described (Ule 1901), this complexity has gradually come into greater focus, though much remains to be learned. Here we review recent progress in AG research and point to specific questions for future investigation that will advance the potential of AGs as a unique source of insight into the evolution of symbiosis and mutualism.

ANT-GARDENS: DEFINITION AND ECOLOGICAL ROLE

ANT-GARDENS ARE INITIATED when arboreal ants collect seeds of specific epiphytic plants and cultivate them in nutrient-rich carton nests that become hanging gardens, each of which may support one or more individual plants from any of several species (Kleinfeldt 1978, Madison 1979, Davidson 1988, Hölldobler & Wilson 1990, Corbara & Dejean 1996, Marini-Filho 1999, Orivel & Dejean 1999, Kaufmann & Maschwitz 2006). The AG interaction was first described in Brazil (Ule 1901) and is still best-known in the Amazon, but similar phenomena have also been documented in Central America (Kleinfeldt 1978,
Longino 1986, Catling 1995) and Southeast Asia (Kaufmann et al. 2001, Kaufmann 2002, Kaufmann & Maschwitz 2006). Though opportunistic ants and plants sometimes occur in AGs, most authors have emphasized that true AG species are only those epiphytes with ant-dispersed seeds, and the ants that actively collect these seeds and incorporate them into carton nests. Further, these species should be essentially AG-restricted, as opposed to opportunistic associates that regularly live independently (Corbara & Dejean 1996, Kaufmann & Maschwitz 2006). Here we follow this strict definition of AG species.

In each region where they occur, AGs comprise a diverse but distinct flora and fauna. Exhaustive compilations of ant and plant species found in Neotropical AGs have been provided by Kleinfeldt (1986) and Kaufmann (2000), including 11 ant species in four subfamilies, and 50 epiphyte species in 12 families. In Table 1 we present only those Neotropical species known or likely to meet the strict definition of AG species. In the Paleotropics, Kaufmann and Maschwitz (2006) identified 67 ant species and 84 epiphyte species participating in AGs, of which 18 and 52 species, respectively, were identified as true AG participants.

AGs are ecologically significant where they occur. This is particularly obvious in the Amazon basin, where the common AG ants Camponotus femoratus (Table 1) and Crematogaster levior (Myrmicinae) form large polydomous (multi-nest) colonies with territories that can occupy up to 39% of the area along forest transects, or, on average, a nest every 14 to 30 linear meters depending on habitat type (Davidson 1988, Marini-Filho 1999, EY unpublished data). Furthermore, Ca. femoratus and Cr. levior are the most frequently
sampled ants in canopy fogging samples and in both terrestrial and arboreal surveys at sites in Perú, Brazil and French Guiana (Jeanne 1979, Wilson 1987, Davidson 1988, Tobin 1991, Dejean et al. 2000, Chapter 2). Finally, AGs are a critical factor predicting the spatial distribution of epiphytes. Not only did four AG-restricted species account for more than half of total epiphyte individuals at a study site in Venezuela, AGs were the single most important habitat for epiphytes generally—both AG-restricted species and opportunists that established on AG carton (Nieder et al. 2000).

In Central America, the nature of ant-epiphyte interactions is less clear. Only for one species pair—the ant *Crematogaster longispina* and the epiphytic plant *Codonanthe crassifolia*—have seed carrying and garden initiation been confirmed (Kleinfeldt 1978). In other apparent AGs, the epiphytic flora is not well described, but sometimes includes the same obligate AG epiphytes found in South America (*Codonanthe* spp., *Peperomia macrostachya*) or their congeners (Longino 1986, Catling 1995, Longino 2008). At least some of the observed epiphyte species appear to be restricted to carton nests (Catling 1995), but Longino (1986) has also postulated a highly generalized interaction in which many vascular epiphytes rely upon the organic material in ant carton for initial colonization until the mass of epiphytes begins to accumulate organic matter independently and can support additional newcomers.

The existence of AGs in Southeast Asia, though proposed as early as 1913, has only recently been confirmed (Kaufmann et al. 2001, Kaufmann & Maschwitz 2006). Their ecological impact in this region is not yet clearly defined, but ant carton provides the only
opportunity for epiphyte establishment in some habitats such as bamboo forest. Kaufmann (2000) further argued that the majority of vascular epiphyte species in the region, excluding ferns and orchids, can be found living in association with ants and have ant-dispersed seeds. The abundance and species richness of the associations suggest that the long-overlooked Paleotropical AGs may indeed play an important role in forest ontogeny and community structure, just as AGs do in the Neotropics.

Throughout this review we will focus primarily upon South American AGs, occasionally pointing to salient comparisons and contrasts with the Central American and Paleotropical systems.

**ANT-GARDENS AS SEED DISPERSAL MUTUALISM**

**NON-AG ANT-SEED MUTUALISMS.**—Seed dispersal mutualisms play a central role in community regeneration and species survival worldwide (Stiles 1992, Christian 2001, Jordano & Godoy 2002). Ants are the only invertebrates that play a significant role in this process, and myrmecochory (seed dispersal by ants) has evolved repeatedly in more than 80 plant families, including some 3000 species, dispersed by hundreds of ant species in dozens of genera and four subfamilies (Gomez & Espalader 1995, Beattie & Hughes 2002). This interaction is mediated primarily by an elaiosome, a lipid-rich seed appendage that, in different plant groups, derives from various anatomical origins (reviewed in Gorb & Gorb 2003). This prompts mutualist ants to retrieve seeds to the nest, where the elaiosome is
consumed and the seed discarded with improved probability of survival and germination; experimental removal of elaiosomes renders the seeds unappealing to mutualist ants (Brew et al. 1989, Beattie & Hughes 2002, Gammans et al. 2005). In turn, elaiosome consumption can improve ant colony productivity (Gammans et al. 2005). Chemical analyses indicate that typical elaiosomes are composed of relatively nonvolatile lipids. Their fatty acid composition more closely resembles that of insect prey than of seeds; the diglyceride 1,2-diolein is usually present, and elicits seed-collecting behavior when contacted by mutualist ants. There is no evidence that ants are attracted to myrmecochorous seeds by olfaction (Brew et al. 1989, Hughes et al. 1994, Sheridan et al. 1996, Gammans et al. 2006).

Myrmecochory is currently understood as a diffuse mutualism, in which, at any given site, dozens of elaiosome-bearing seed species can appeal to several ant species (e.g. Beattie & Hughes 2002, Garrido et al. 2002, Bronstein et al. 2006, Zhou et al. 2007). Nevertheless, some evidence indicates that the interaction is not necessarily evenly diffuse, that is, one or few keystone ant mutualists may have a disproportionately large effect on plant fitness (Bronstein et al. 2006, Giladi 2006, Gove et al. 2007). As a way for seeds to arrive at fertile microsites and to escape the parent plant, fire or granivores, myrmecochory is common and fundamental to community organization in subtropical fire-dominated sclerophyll habitats and temperate mesic forests (Westoby et al. 1991, Christian 2001, Beattie & Hughes 2002, Gorb & Gorb 2003).

In tropical habitats, however, the mechanisms and importance of ant-seed interactions are poorly understood. Interactions resembling typical myrmecochory—that is,
dispersal of elaiosome-bearing seeds to terrestrial ant nests—have, to our knowledge, been reported only in four tropical rainforest species, a major underrepresentation of this dispersal strategy relative to the diversity of tropical plants (Horvitz & Beattie 1980, Passos & Ferreira 1996, Pfeiffer et al. 2004). Evidence suggests that ants do play an important role in tropical seed viability and dispersal, but most such interactions appear to be opportunistic. With or without relocating the seeds, generalist and fungus-gardening ants may clean adhering fruit pulp from fallen vertebrate-dispersed seeds, improving their survival rates and protecting them against fungal attack (Oliveira et al. 1995, Leal & Oliveira 1998, Ohkawara & Akino 2004, Christianini et al. 2007). Seeds cached but not eaten by litter- and twig-nesting granivores, and lipid-rich seeds that are commonly dispersed and cleaned by carnivorous ants, also have an improved germination rate relative to seeds that had no interaction with ants (Roberts & Heithaus 1986, Kaspari 1993, Levey & Byrne 1993, Pizo & Oliveira 2001, Christianini et al. 2007). Though high seed moisture and lipid content increase the probability of interaction with ants (Pizo & Oliveira 2001, Fornara & Dalling 2005), none of these interactions appears to be specialized or species-specific (Pizo & Oliveira 2000).

**Basis of the AG Ant-Seed Interaction.**—AG seed dispersal differs markedly from both typical myrmecochory and from the opportunistic ant-seed interactions known from tropical forests. AG participants exhibit a higher degree of species-specificity than these other interactions—or in fact than most seed dispersal mutualisms of any type (Chapter 2). The
ants and epiphytes in Table 1 are rarely found living independently, and this pattern of “few-to-few” specificity arises directly from the seed-dispersal step of the interaction, in which AG ants reject most non-AG seeds tested (Davidson 1988, EY pers. obs.), and most non-AG ants reject AG seeds. Specifically, three out of four non-AG ant species rejected seeds of three AG seed species (Davidson 1988), and only three of 70 ant species detected along bait transects retrieved *P. macrostachya* seeds; of the three species, *Ca. femoratus* was responsible for 84% of seed removal (Chapter 2).

The specificity of the interaction suggests that general nutritional rewards, which should be utilized by multiple ant species, are probably not the sole motive for seed-collecting. Indeed, few AG species bear elaiosomes; among the Neotropical species in Table 1, only *Codonanthe* species have a fleshy aril that clearly resembles the elaiosome of typical myrmecochores—but the chemistry of these arils has never been directly analyzed for elaiosome-like composition (Kleinfeldt 1986, Davidson 1988, EY pers. obs.). Some AG species (e.g., *A. gracile*, *A. longifolia*) produce seeds encased in a sweet gelatinous matrix that is distinct from the surrounding fruit, while others (*M. ulei*) may simply retain fruit juice or pulp (Davidson 1988). *P. macrostachya* seeds are covered in a cream-colored aril, and also bear a sticky oil gland, both of which are sometimes assumed to serve as food rewards (Madison 1979, Davidson 1988, Orivel & Dejean 1999). To our knowledge, no direct observations confirm that either aril or oil gland is actually utilized by the ants; in fact, *P. macrostachya* seeds with intact arils are commonly embedded in *Ca. femoratus* nest carton, suggesting that they are not eaten (EY pers. obs.). Davidson (1988) noted the presence of
AG seeds in *Ca. femoratus* and *Cr. levior* brood chambers and carton walls, where larvae may have fed upon putative food rewards and did not damage seeds. Nutritional value of these possible rewards on AG seeds has never been measured.

Regardless of their actual value and use, evidence suggests that the putative food rewards are not essential to AG ant-seed interaction. Orivel and Dejean (1999) removed all apparent nutritional rewards from the seeds of *Ae. mertensii*, *An. gracile*, *Co. calcarata*, and *P. macrostachya* and presented these to the AG ants *Ca. femoratus* and *P. goeldii*. For *P. goeldii*, seed preferences were unchanged by this manipulation; *Ca. femoratus* also still retrieved the seeds, though its preference for *Co. calcarata* decreased after removal of the aril. Furthermore, *Ca. femoratus* retrieved seeds of the AG epiphyte *F. paraensis*, and continued to ignore the seeds of a non-AG *Ficus* species, even after both sets of seeds had passed through a vertebrate digestive system, which would presumably have thoroughly removed any adhering nutritional rewards (Davidson 1988).

Similarly, in Paleotropical AGs, few of the participating epiphytes provide food rewards on their seeds, seeds were still accepted by AG ants after passage through a bird digestive system, and starved ants demonstrated less interest in the seeds than did well-nourished ones (Kaufmann *et al.* 2001, Kaufmann 2002). AG seeds with adaptations for primary dispersal by wind were taken by AG ant species, but by only one of six non-AG ant species tested. On the other hand, AG epiphyte seeds that originated from bird-dispersed fruits were widely accepted by both AG and non-AG ants, suggesting that this subset of seeds may, in fact, employ a more general mechanism for recruiting ants (Kaufmann 2002).
The observations that many ants reject AG seeds, and that AG ants collect the seeds independently of nutritional rewards, point to the possibility that the interaction is mediated by non-nutritive semiochemicals. Seidel et al. (1990) were the first to investigate possible chemical cues for the ant-seed interaction by analyzing the volatile composition of seeds from 10 AG-restricted epiphyte species in southeast Perú. (Figure 1, Table 2). Nine of the 10 species contained the compound methyl-6-methylsalicylate (6-MMS), and all released overlapping blends of four other phenolic volatiles (Figure 1, Table 2) (Davidson et al. 1990, Seidel et al. 1990). When synthetic seed compounds were presented individually and in blends to Ca. femoratus on artificial "seeds" (molecular sieves), ants responded to seed compounds and their structural relatives with excitement and/or alarm, but they carried the artificial seeds rarely and inconsistently (Davidson et al. 1990). The presence of 6-MMS has been confirmed in eight AG seed species, and an additional four compounds, all terpene and terpenoid volatiles, have been found in all or most AG seeds analyzed but not non-AG congeners (Table 2, Chapter 4). But again, seed-carrying assays that included these compounds were inconclusive and a blend of all five compounds failed to attract Ca. femoratus over distance (Chapter 4). Thus the approach of identifying ant-attracting chemical blend common to all AG seeds, while tantalizing, has so far proven unsatisfactory.

A different set of experiments with seed extracts, rather than synthetic compounds, has nevertheless confirmed that chemical cues are necessary and sufficient to attract Ca. femoratus and elicit seed-carrying behavior. When solvent extracts of P. macrostachya, A. gracile, F. paraensis, or C. uleana were applied to non-AG seeds that the ants ordinarily
ignore, *Ca. femoratus* regularly retrieved these "artificial" AG seeds, collected very few solvent-treated controls, and demonstrated a positive dose-response to *P. macrostachya* extract (Youngsteadt et al. 2008, Chapter 4). Solvents of differing polarity were required to obtain behaviorally active extracts of the three seed species, and different chromatographic fractions of the three extracts elicited seed carrying in *Ca. femoratus*, indicating that these three species use different classes of chemical cues to elicit the same ant response. The identity of these cues has yet to be discovered.

Youngsteadt et al. (2008) further demonstrated that volatile compounds from *P. macrostachya* seeds serve as olfactory attractants for *Ca. femoratus*—but do not themselves mediate seed-carrying behavior, which probably depends upon contact chemical cues. An olfactory attraction step is unknown in other ant-seed interactions (Sheridan et al. 1996). *P. macrostachya* seeds and extracts were preferred over controls in an olfactometer assay, as was a blend of five electrophysiologically active compounds identified from *P. macrostachya* extract. Although this blend included some of the same compounds detected in all AG seeds, it also included some compounds unique to *P. macrostachya*. A separate blend of only the common compounds was not attractive, further discrediting the hypothesis that a universal AG seed signature may mediate the interaction (Chapter 4). Nevertheless, the most abundant component of the attractive blend, geranyl linalool, was indeed shared among all AG seeds tested; interestingly this compound also occurs in the defensive secretions of *Reticulitermes* termite soldiers, and is toxic to many ant species (Lemaire et al. 1990).
Solvent extracts of Paleotropical AGs were also sufficient to elicit seed-carrying in most AG ants in that region, while some species required a combination of chemistry and the correct seed size (Kaufmann 2002). The identities of the specific compounds that elicit seed-carrying were not determined. Primarily bird-dispersed and wind-dispersed seeds probably used different chemical strategies, since the former were widely attractive to many ants while the latter were not. Within each category, it is unclear whether the different seed species produced similar or different compounds to elicit retrieval by AG ants. Kaufmann (2002) speculated that olfaction has no role in the Paleotropical AGs.

Despite progress, much remains to be learned about the species specificity and chemical basis of the AG ant-seed interaction. The specific compounds that elicit seed-carrying have yet to be identified in any AG species, and no species other than *P. macrostachya* has been tested for volatile attractants. (Preliminary tests with *A. longifolia* seeds suggest that they too are attractive to *Ca. femoratus* in the olfactometer (Youngsteadt, unpublished data) and most Neotropical AG seeds have a strong aroma.) What role, if any, the universal AG seed volatiles play in the interaction is unknown. A better understanding of the chemical basis of the AG mutualism could provide insight into the origins of the interaction, but until the responsible compounds are identified, it is impossible to suggest whether their production by seeds is simply a pre-adaptation that facilitated the evolution of AGs, whether they may in fact be specialized metabolites that were modified in response to selection pressure by ants or whether, as suggested by Seidel *et al.* (1990) they may be the metabolic products of symbiotic microbes.
CONSEQUENCES OF THE ANT-SEED INTERACTION.—Perhaps the most glaring omission from studies of AGs is an examination of seed and pollen movement in AG plants. AG ants often collect seeds directly from their gardens and from the ground beneath, transferring them back to the nest carton (Davidson 1988, EY pers. obs.). Further, Youngsteadt et al. (Chapter 2) showed that, at a study site in southeast Perú, vertebrates have little role in *P. macrostachya* seed fate once the seeds fall to the forest floor. This would seem to severely limit opportunities for long-distance dispersal to new sites, but several observations suggest that vertebrates may disperse AG seeds directly from the parent plant. Davidson (1988) reported that birds, bats and monkeys feed on the fruits of *An. gracile, Ph. megalophyllum, E. phyllanthus* and *F. paraensis*, that *Pe. macrostachya* seeds can be found in bat droppings, and that *F. paraensis* seeds were still collected by *Ca. femoratus* after passing through the digestive system of a bat. The sticky *Pe. macrostachya* seeds are also thought to be dispersed by adhering to passing vertebrates (Madison 1979). Observations of tanagers (*Tangara schrankii*) nesting in southeast Perú also revealed *Peperomia* sp. seeds in the birds' droppings, and the feces were collected by foraging *Ca. femoratus* from a nearby AG (Van Houtan & Alvarez-Loayza 2006). Given that vertebrates use AG seeds, that ants use vertebrate feces, and that ants recognize AG seeds even after "digestion," it seems likely that ants may also remove AG seeds from vertebrate feces after longer-distance dispersal. Further, though many AG species are capable of self-pollination, it is unclear to what extent they do so, and whether pollinators are deterred by ants (Madison 1979, Davidson 1988).
Quantifying the importance of pollinators and vertebrate dispersers in maintaining long-distance gene flow in AG plant communities is critical to understanding the consequences of the AG interaction from an evolutionary perspective. Understanding the roles of pollinators and vertebrate dispersers also has ramifications for conservation, since the stability of AG species might depend upon fairly cryptic interactions with organisms that are rare or are more directly susceptible to climate change or habitat fragmentation than the AG species themselves.

In addition to careful field observations, genetic studies should be undertaken to address these issues. Actual self-pollination rates could be determined by comparing parental and offspring plant genotypes; this should be undertaken at multiple sites, and would be particularly informative if coupled with the same measures for non-AG congeners and/or for the same species living outside of AGs. An analysis of population genetic structure at a range of spatial scales would further suggest the extent of long-distance seed- and/or pollen movement in these species and would indicate the extent to which ant-dispersal influences population structure—and hence evolutionary processes—in the AG epiphytes.

The influence of seed dispersal mutualisms on plant population structure has only recently begun to be addressed in any system, and three studies have found evidence that seed-dispersal by ants can indeed limit gene flow and lead to local population genetic structure on a small spatial scale, though the effect can vary by site (Peakall & Beattie 1995, Maeyama & Matsumoto 2000, Zhou et al. 2007). One of these studies addressed an AG-like
symbiosis between the ant *Dolichoderus* sp. (Dolichoderinae) and the ant-house epiphyte *Anthorrhiza caerulea* (Rubiaceae), endemic to Papua New Guinea. The ants live within hollow plant tubers, and a single colony occupies multiple plants connected by carton runways. The ants collect *A. caerulea* seeds, store them within the tubers, and embed them in newly constructed carton, where the plants become established (Maeyama & Matsumoto 2000). Using RAPD markers, Maeyama & Matsumoto (2000) identified boundaries of seven polydomous ant colonies, and found that all *A. caerulea* plants within a given colony were more closely related to one another than they were to plants in other colonies. A similar result might be expected in other AG systems, depending upon the prevalence of long-distance dispersal by vertebrates and the ability of ants to retrieve seeds after vertebrate dispersal.

**ANT-GARDENS AS ANT-PLANT SYMBIOSES**

AGS ARE UNIQUE AMONG SEED-DISPERSAL MUTUALISMS in that the ant and plant partners remain associated throughout their life histories. Whereas other animal-dispersed seeds are abandoned after the removal of edible portions, AG ants and plants live in an obligate symbiosis well past the act of seed dispersal. (Only in human agriculture is there a comparably long-term interaction between disperser and dispersed.) During this extended relationship, benefits accrue to both ant and plant partners. For the ants, this includes structural stability of carton nests and, to some degree, food resources. For the plants,
benefits include directed seed dispersal to fertile carton substrate and, perhaps, defense against herbivores. Possible costs to participants have not been measured.

The abundance of the AG ants *Ca. femoratus* and *Cr. levior* throughout the Amazon basin (see above) has been attributed to their mutualism with epiphytes, which frees them to build large stable nests in resource rich microhabitats, independently of pre-existing nest holes and substrates (Wilson 1987, Davidson 1988). The epiphytes have this effect because they draw water out of, and perhaps shelter, the nest carton; defoliated *Ca. femoratus* nests became saturated and disintegrated during the rainy season (Yu 1994). The role of epiphytes in nest stability of Central American AGs is unclear. Kleinfeldt (1978) suggested that *C. longispina* nests were stable even without epiphytes, though Longino (2008) described *C. longispina* carton as friable and easily disrupted. Similarly, experimental data are lacking for Paleotropical AGs, but many of these epiphyte species provide domatia for the resident ants, an interaction appreciated as providing important nest space for ants even before the interaction was also recognized a type of AG (Kaufmann & Maschwitz 2006).

AG ants may derive some nutritional benefit from their gardens in the form of extrafloral nectaries, fruits, and honeydew-producing hemipterans, but the large colonies of *Ca. femoratus* and *Cr. levior* forage well beyond their nests, feeding especially on plant and hemipteran exudates and also hunting and scavenging for protein (Davidson 1988) (EY pers. obs.). Most paleotropical AG ants maintain hemipteran populations on the host tree beneath the carton nest (Kaufmann & Maschwitz 2006). In contrast, *C. longispina* in Costa Rica
relied almost exclusively upon floral and extrafloral nectar from its symbiotic *Co. crassifolia* (Kleinfeldt 1978).

Hypothesized costs to the ants of maintaining AGs include eventual crowding by roots of some epiphyte species (Davidson 1988) and shading of the nest, which could slow temperature-dependent brood development (Longino 1986) – though others have suggested that shading would beneficially insulate the colony from daily temperature extremes (Belin-Depoux *et al.* 1987, Kaufmann 2002). None of the proposed costs has been experimentally tested.

For AG epiphytes, the clearest benefits are access to fertile growth substrate (carton) and highly directed seed dispersal to such substrate—which also, crucially, diverts epiphyte seeds away from the ground where they are doomed. In lowland forests where substrate and nutrient availability are limiting for epiphytes, *Ca. femoratus* carton, constructed of various organic materials including vertebrate feces, provides a porous texture and enriched N and P content relative to adjacent soil and carton built by termites, non-AG *Pheidole* ants, and *Azteca* ants that only occasionally hosted AG epiphytes (Blüthgen *et al.* 2001). AG carton constructed by unidentified ant species was also richer in nutrients than surrounding substrates such as soil, bark, and a fern root ball (Belin-Depoux *et al.* 1987, Benzing 1991). In Costa Rica, *Co. crassifolia* developed roots where ants built carton around leaf nodes, and individuals with ants grew faster than those without ants during a five-month study period (Kleinfeldt 1978). In Paleotropical AGs, Kaufmann (2002) compared carton of six AG species to that of a non-AG ant, termite carton, soil and bark. Here, AG carton had high
water-storage capacity, and although AG carton composition was highly variable, it nearly always contained a higher concentration of phosphate and/or nitrate than other substrates. Unfortunately, there has been no similar attempt to make a uniform assessment of carton nutrient quality across the several Neotropical AG ant species. Such a comparison would suggest whether there may be selection upon the plants to "prefer" some ant partners over others—though additional factors, such as degree of herbivore protection and light level of preferred nest sites, would also influence the overall suitability of an ant species as a partner.

Even *Ca. femoratus* gardens occur at an enormous range of heights, from little more than a meter above the ground to tens of meters high in the canopy. Different AG species are over-represented in different canopy strata, with *Pe. macrostachya, Ph. megalophyllum*, and *M. ulei* more common in lower strata and therefore considered shade-tolerant, while *An. gracile, Co. uleana, F. paraensis, E. phyllanthus*, and *Ae. longifolia* are overrepresented in higher canopy strata (Davidson 1988). This is typical of epiphytes generally; species are usually most abundant in characteristic canopy strata that confer optimal levels of light and humidity. Thus, AG seeds run the risk of being "planted" in nests with non-optimal light levels—but any AG certainly presents a better opportunity than the forest floor (Chapter 2).

Finally, AG ants may provide their resident epiphytes with some protection against herbivores, though thorough tests are lacking, and, again, little effort has been made to compare the defensive capabilities of the various gardening ant species, or whether they defend all AG plant species equally well. Nevertheless, unpublished data indicate that *Ca. femoratus* and *Cr. levior* provide significant protection against insect herbivores of *P.*
macrostachya, C. uleana, M. ulei, and F. paraensis (Davidson & Epstein 1989). Further, both Ca. femoratus and Cr. levior continuously patrolled garden epiphytes, discovered experimental leaf damage within 1 minute, and ants quickly accumulated on damaged leaves (Vantaux et al. 2007). In contrast, less than half of experimentally damaged host tree leaves were discovered by ants after 30 minutes—though it is unclear whether this is due simply to lower ant traffic farther from the center of the nest as opposed to specialized response to AG plants. Even so, the authors argue that constant patrolling of AG leaves by ants is likely to deter herbivores, and that ant response to plant wounds would further diminish herbivory. Kleinfeldt (1978) reported that C. calcarata appeared to suffer the same amount of herbivore damage when growing with or without ants, further noting that C. longispina made no attempt to displace chrysomelid beetles feeding upon the leaves. The role of Paleotropical AG ants in plant defense has not been investigated experimentally, and anecdotal observations suggest that, again, defensive ability varies among the ant species (Kaufmann 2002).

Potential costs to AG plants are unclear. Until the chemical basis of the seed-dispersal is determined, it will be impossible to know how much the plants invest in recruiting ants for dispersal. Most AG plants also produce extrafloral nectar and/or food bodies; these are produced at some unknown cost, and appear to promote patrolling of leaves by ants, as well as carton construction around parts of the plant where additional roots can grow (Kleinfeldt 1978, Vantaux et al. 2007). Ants might limit gene flow and outbreeding by excluding pollinators and by incorporating seeds into carton very near the
parent plant. Given limited space in the carton and low overall probability of seedling survival (Chapter 2), ants probably also promote intense competition among closely related individual plants.

Until the costs and benefits of the AG interaction—and how these vary in time, space and across partner species—are clarified, the contribution of AGs to our understanding of mutualism will remain limited. Better understanding of the fitness effects of the interaction upon each participant, however, would provide an unprecedented opportunity to examine the factors that led several unrelated species on two different continents to converge upon a very similar obligate mutualism.

**ANT-GARDENS AS PARABIOSES**

Many AGs house more than one ant species in a poorly understood interaction termed parabiosis. In this relationship, two or more ant species share a nest and forage together on common trails, while maintaining brood separately and independently inside the nest (Forel 1898). Parabiosis has been reported among at least 18 ant species living together as 13 different pairs, and at least one trio, in the South American and Asian tropics (Table 3); in most cases, it appears to be a facultative interaction (but see below) (Mann 1912, Weber 1943, Swain 1980, Orivel et al. 1997, Lenoir et al. 2001, Kaufmann & Maschwitz 2006, Menzel et al. 2008). The phenomenon presents a puzzle in social insect biology, since colonies of ants and other social insects are typically closed societies that do not tolerate
other species or even conspecific members of other colonies. Known exceptions include parasites that gain access to social insect colonies by synthesizing or acquiring the cuticular hydrocarbons used for nestmate recognition cues in the host species, or by emitting "propaganda" chemicals that induce panic or confusion in the host (Lenoir et al. 2001). This appears not to be the case in parabiosis, and even though some parabiotic species such as *Ca. femoratus* and *Cr. levior* are spectacularly abundant, their interaction remains enigmatic.

The behavioral mechanisms that accommodate cohabitation of parabiotic ant species are unknown, as are the costs and benefits that might motivate such associations. The Neotropical AG ants are the most-studied parabiotic species, and here we review what is known about parabiosis in AGs as well as other ant partnerships.

At different sites, 77% to more than 98% of *Ca. femoratus* AGs also house *Cr. levior* (Davidson 1988, Orivel et al. 1996, 1997, EY unpublished data). *Cr. levior*, by definition, is an obligate parabiotic partner of *Ca. femoratus* while closely related and morphologically overlapping *Crematogaster* found living alone or in parabiosis with other ant species are assigned to the species *C. carinata* (Longino 2003). Not all observations agree, however; researchers working in French Guiana noted that a single *Crematogaster* species, reported first under the name *C. limata parabiotica* and then under the name *Cr. levior*, is found in parabiosis not only with *Ca. femoratus* but also with *O. mayi* and occasionally *P. goeldii*, but also lives independently and maintains its own AGs about 14% of the time (Orivel et al. 1996, Orivel & Dejean 1999, Vantaux et al. 2007). There may still be some confusion as to how many and which *Crematogaster* species are involved, which could also explain

It is unclear whether and how much *Cr. levior* contributes to nest construction when living with another species. When *Cr. levior* lives alone, its nests are smaller, and, though it shows interest in AG seeds, it has never been directly observed to carry them (Davidson 1988, Orivel & Dejean 1999, Dejean *et al.* 2000, Chapter 2). In French Guiana, however, there was a trend that gardens housing parabiotic ants were intermediate in epiphyte composition between those that housed each species individually, suggesting that *Cr. levior* might contribute to garden construction and seed collecting (Orivel *et al.* 1996 but see Dejean *et al.* 2000). These observations did not explicitly control for possible confounding factors such as garden age or microhabitat, which might influence both floristic composition and the presence of parabiotic ants. Dejean *et al.* (2000) found that young *Ca. femoratus* gardens rarely housed *Cr. levior*, while older ones did.

Hypothesized advantages of parabiosis to *Cr. levior* include nest space in resource-rich habitat patches, and protection from enemies, particularly vertebrates, by the larger and more pugnacious *Ca. femoratus*. Longino (2003) refers to unpublished results by D. Davidson indicating that *Cr. levior* lacks chemical defenses typical of the genus, also reflected in absence of gaster-raising behavior upon disturbance. Chemical defense and gaster-raising are retained in the closely related *C. carinata* that lives alone or in parabiosis with other species—such as *Dolichoderus debilis*, which itself has timid behavior and diminished chemical defense relative to other *Dolichoderus* species (Longino 2003). These
results are tantalizing, and suggest that loss of defensive ability may be an evolved consequence of parabiosis that should be confirmed and assessed in other parabiotic pairs.

If one species benefits from protection with a more aggressive species, what costs or benefits accrue to the defender? The only experimental evidence available to date also indicate a benefit to Ca. femoratus, in that Cr. levior is often the first to encounter baits of sugar, protein, or AG epiphyte seeds, which the larger species then shares or usurps, often after following Cr. levior recruitment trails (Swain 1980, Davidson 1988, Orivel & Dejean 1999, Vantaux et al. 2007). An additional hypothesized benefit to Ca. femoratus includes access to honeydew from adult hemipterans that were tended during their small, less profitable stages by Cr. levior. Finally, both species are known to participate in defense of the AG epiphytes by patrolling leaves, particularly where these have been injured, a result that has been interpreted as mutually beneficial for both ants and epiphytes (Vantaux et al. 2007).

Because it is unusual for multiple ant species to peacefully share the same nest, several studies have addressed nestmate recognition behavior in parabiotic species. These studies have yielded widely varied results in different species (Table 3), ranging from acceptance of any member of the partner species regardless of colony of origin, to aggression toward any non-nestmate of either species (Orivel et al. 1997, Lenoir et al. 2001, Errard et al. 2003, Menzel et al. 2008, Youngsteadt et al. unpublished data). The chemical recognition systems underlying the variable nestmate recognition behavior are also poorly understood. Unlike many social parasites of ant nests, all Neotropical parabiotic species
examined (including *Ca. femoratus*, *Cr. levior*, *P. goeldii* and *O. mayi* as well as non-AG species) had species- and colony- specific cuticular hydrocarbon profiles (Orivel *et al*. 1997, Lenoir *et al*. 2001, Errard *et al*. 2003, Youngsteadt *et al*. unpublished data). This suggests that parabiotic ants tolerate one another not because of mistaken identity, but because they simply learn to identify and recognize one another at the species- or colony- level (Orivel *et al*. 1997, Errard *et al*. 2003). In contrast, a Southeast Asian parabiotic pair *Camponotus rufifemur*/*Crematogaster modiglianii* has no detectable cuticular hydrocarbons, and the two species instead share a common cholesterol-like cuticular compound, and demonstrate inconsistent ability to distinguish between nestmates and non-nestmates (N. Blüthgen, pers. comm.).

Unfortunately, though cuticular hydrocarbon composition of AG ants varies between nests and between colonies (Orivel *et al*. 1997, Youngsteadt *et al*. unpublished data), no study has confirmed the behavioral relevance of the cuticular substances studied as nestmate recognition cues in any of the parabiotic ant species. Although hydrocarbons are indeed the relevant substance in many other ants, it should not be assumed, given the puzzling nature of the parabiotic interactions, that hydrocarbons alone mediate recognition in these species.

Further, other factors likely to influence nestmate recognition, including colony structure and population genetic structure, have not been clearly defined in most parabiotic species (Table 3). All Neotropical AG species can be polydomous (Table 3), and genetic homogeneity among clustered nests of *Ca. femoratus* and *Cr. levior* has been confirmed using microsatellite markers (Booth *et al*. 2008b, Booth *et al*. 2008a, Youngsteadt *et al*.).
unpublished data). Moreover, colony boundaries of the two species largely coincided, and neighboring colonies were only distantly related (Youngsteadt et al. unpublished data).

As a phenomenon that has evolved repeatedly in tropical ant species, and as a challenge to understanding of social insect behavior, parabiosis is worth solving. Whether parabiosis really means the same thing in all observed species pairs, or whether parabiotic associations are superficially similar but originated from different selective pressures and different behavioral mechanisms, remains unclear. In order to make sense of the phenomenon, the following questions need to be addressed: What are the costs and benefits of parabiosis to each participating species? This must be more thoroughly and directly assessed, beyond the short-term or anecdotal observations currently available. What sequence of events occurs during the founding of a parabiotic colony, i.e. how and at what stage do the two species become associated, and what behavioral interactions take place at these earliest stages, such as when a queen of one species enters the nest of the other species? What chemical cues do participating species use for nestmate recognition?

EVOLUTION OF THE AG SYMBIOSES

Possible scenarios for the evolution of AG ant-epiphyte interactions have been discussed at length and always conclude with the same impasse: lack of information (Davidson 1988, Davidson & Epstein 1989, Davidson et al. 1990, Seidel et al. 1990, Kaufmann 2002). One proposed sequence is that ants that built nests in or around epiphytes,
or tended their extrafloral nectaries, may have also deterred vertebrate seed dispersers. Such ants would find themselves with an abundance of nearby seeds, some of which may have been sweet, sticky, etc. (Davidson 1988, Davidson & Epstein 1989). Even without deterring vertebrate dispersers, ants may have collected epiphyte seeds that were generally attractive because of sugars or lipids (Longino 1986, Davidson & Epstein 1989). This seems to still be the case in the primarily bird-dispersed Paleotropical AG species, which are taken by many non-AG ants as well as the AG ants. But in epiphyte species taken only by AG ants, addition of other compounds, such as the phenolic and terpenoid volatiles found on Neotropical AG seeds, might modulate ant response to the generalized attractants and deter non-AG ants (Kaufmann 2002). Finally, rather than layering compounds accepted by many ants with compounds accepted by few ants, seeds may only have the narrowly accepted compounds (Kaufmann 2002).

Unfortunately, no new evidence has been brought to bear upon these possibilities since they were last discussed. Here we suggest three approaches that could shed light on the origins and possible coevolution of AG ants and plants. Phylogenies are becoming available for some of the participating species, introducing the possibility that precursors or transitional states to the AG habit, or traits that set AG species apart from their closest non-AG relatives, might soon be identified. Molecular phylogenies have recently been published for Peperomia, Ficus, and Philodendron, each of which places the AG species in well-supported clades (Rønsted et al. 2005, Wanke et al. 2006, Gauthier et al. 2008). A new phylogeny for Aechmea, based on morphology, has also been proposed, and includes the AG
species *A. longifolia* (de Faria et al. 2004). These new resources should help target species in which ant response to seeds, seed chemistry, mode of seed dispersal, requirements for seedling establishment, foliar chemical defenses, and the presence of extrafloral nectar and food bodies should all be evaluated in order to sketch a picture of the evolution of these characters relative to the AG habit.

A forthcoming revision of *Camponotus* (W. Mackay pers. comm.) and a recent molecular phylogeny for *Odontomachus* (Spagna et al. 2008) may similarly point toward which ant species should be compared to AG ants in terms of seed-collecting behavior, nesting habit and, in the case of carton nests, carton composition and durability.

In addition to comparing close relatives of AG species, further insight might also be gained by examining the life histories of AG epiphytes with ranges larger than those of the most assiduous gardening ants. Several species restricted to AGs in South America also occur in Central America (e.g., *Ae. tillandsioides, An. gracile, C. crassifolia, C. uleana, E. phyllanthis, F. paraensis, M. ulei, Pe. macrostachya*) (Croat 1978). A close examination of whether and how these species form ant associations in this region might reveal transitional forms of ant-epiphyte association that in the Amazon have been lost or crowded out by dominant seed-collecting species like *Ca. femoratus*.

Finally, the question of coevolution in the AG system has nagged at nearly every biologist engaged in AG research. Yet every trait of AG ants and plants that may facilitate the AG habit—from seed chemistry to ant aggression to carton composition—can also be plausibly explained as a preadaptation requiring no special evolutionary modification for
AGs (Longino 1986, Davidson 1988, Seidel et al. 1990, Kaufmann 2002). Perhaps the only hope for settling this question would be to undertake the rather impractical task of examining geographic variation in AG-related traits (e.g. Thompson 2005). Relevant questions include: Does Ca. femoratus (or another AG ant species) prefer AG seeds from its own locality to seeds of the same species originating from distant sites? If so, does the preference correlate to any detectable difference in physical or chemical characteristics of the seeds? Are ant preference and the variable seed traits both under genetic control, i.e., do differences persist in a common environment? Given the wide geographic range of the AG interaction, and the likelihood of limited gene flow throughout the region, such local coadaptation is plausible.

Similar evidence might be sought in a comparison of same-species seeds from regions where different AG ant species dominate. Sites where Ca. femoratus is rare but P. goeldii and O. mayi are common have been described, as have sites where the three co-occur (Marini-Filho 1999, Dejean et al. 2000). These ant species are known to collect the same AG seed species, but in different order of preference (Orivel & Dejean 1999). Furthermore, there is some evidence that, at some sites, garden-founding may in fact occur primarily when ants occupy already-established epiphytes of the same species that elsewhere only thrive when their seeds germinate in carton (Belin-Depoux et al. 1987, Cedeño et al. 1999, VanDunné 2001, G. Mathieu pers. comm.). If such observations were confirmed, seed traits should be compared between regions where ant-dispersal is necessary for survival and regions where it is not. Given sufficient isolation between plant populations, different ant
species or communities might select for different seed traits, giving rise to detectable intraspecific variation in the plants. This kind of evidence—or lack of such evidence—would be valuable in building a case for or against coevolution in the AG interaction.

A study with similar aims was conducted for the European myrmecochorous herb *Helleborus foetidus*, in which seed and elaiosome dimensions and ant community composition were assessed at nine different sites in Spain (Garrido *et al.* 2002). No clear relationship emerged, with seed- and ant- size well matched at some localities and not at others. The chances of detecting such a relationship may be better in the AG system, where there are fewer ant species dispersing the seeds (17 ant species collected *H. foetidus* in Spain), and where the geographic range is larger.

**CONCLUSION**

**ANT-PLANT INTERACTIONS**, particularly ant-plant protection mutualisms and seed-dispersal by ants, have contributed much to current understanding of the evolution and ecology of mutualism by providing fascinating case studies of the circumstances under which mutualisms form, evolve, specialize, coevolve, and thwart cheaters (Bronstein 1998, Bronstein *et al.* 2006). None of these aspects is fully understood, and the AGs are a promising system to further investigate such questions. They are ecologically important mutualisms that combine elements of protection and seed-dispersal interactions, are intermediate in species-specificity, and have evolved repeatedly in numerous ant and plant
taxa, some of which have been recently organized in extensive morphological and molecular
phylogenies. There has been progress documenting benefits of the AG interaction to
participating species, examining the chemical basis of the ant-seed interaction, and also
demonstrating the species-specificity of the ant-seed interaction. In order for this rich system
to contribute further to our understanding of mutualism, however, several aspects of AGs
require further study. Longer-term studies of costs and benefits of the interaction,
particularly the consequences of the AG mutualism on seed and pollen dispersal and
population genetic structure of the plants, would be especially useful. Assessment of
geographic variation in AG-related traits and their possible coevolution, and comparison of
AG-related traits in closely related non-AG species would also provide important insight
into the evolution and possible coevolution of the interaction.

ACKNOWLEDGMENTS

We thank F. Gould, J. Silverman and E. Vargo for review and discussion of this manuscript,
and G. Mathieu and K. Van Houtan for sharing their own observations Neotropical AGs.
Our work in this system has been supported by a National Science Foundation Predoctoral
Fellowship, a U.S. Department of Education GAANN Biotechnology Fellowship, an
Amazon Conservation Association Graduate Research Fellowship (EY), and the Blanton J.
Whitmire Endowment (CS), and was facilitated by the Instituto Nacional de Recursos
Naturales (INRENA) in Perú.
LITERATURE CITED


fruits and seeds in cerrado vegetation in southeast Brazil. Biotropica 30: 170-178.
Geranyl linalool (diterpene alcohol): An insecticidal component of pine wood and 
termites (Isoptera: Rhinotermitidae) in four European ecosystems. Journal of 
Chemical Ecology 16: 2067-2079.
LEVEY, D. J., and M. M. BYRNE. 1993. Complex ant-plant Interactions: Rain-forest ants as 
secondary dispersers and post-dispersal seed predators. Ecology (New York) 74: 
1802-1812.
LONGINO, J. 2003. The Crematogaster (Hymenoptera, Formicidae, Myrmicinae) of Costa 
MADISON, M. 1979. Additional observations on ant-gardens in Amazonas. Selbyana 5: 107-
115.
MAEYAMA, T., and T. MATSUMOTO. 2000. Genetic relationship of myrmecophyte 
(Anthorrhiza caerulea) individuals within and among territories of the arboreal ant
(Dolichoderus sp.) detected using random amplified polymorphic DNA markers.


Symbiosis as a Source of Evolutionary Innovation, pp. 1-14. The MIT Press,

Cambridge, MA.

MARINI-FILHO, O. J. 1999. Distribution, composition, and dispersal of ant gardens and

tending ants in three kinds of central Amazonian habitats. Tropical Zoology 12: 289-

296.


MENZEL, F., K. E. LINSENMAIR, and N. BLÜTHGEN. 2008. Selective interspecific tolerance in

NIEDER, J., S. ENGWALD, M. KLAWUN, and W. BARTHLOTT. 2000. Spatial distribution of
vascular epiphytes (including hemiepiphytes) in a lowland Amazonian rain forest
(Surumoni crane plot) of southern Venezuela. Biotropica 32: 385-396.

OHKAWARA, K., and T. AKINO. 2004. Seed cleaning behavior by tropical ants and its anti-


Mycocepurus goeldii ants (Attini) facilitates germination in Hymenaea courbaril


Table 1. Ant species that construct AGs, and epiphyte species that produce seeds dispersed by these ants, that are restricted to AGs, and/or are frequently reported in AGs.

### Ants observed to construct carton and collect seeds

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formicinai</td>
<td>Camponotus femoratus (Fabricius)</td>
<td>6, 11, 14</td>
</tr>
<tr>
<td>Dolichoderinae</td>
<td>Azteca traili Emery</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Azteca spp</td>
<td>10, 15</td>
</tr>
<tr>
<td>Myrmicinae</td>
<td>Crematogaster longispina Emery</td>
<td>2</td>
</tr>
<tr>
<td>Ponerinae</td>
<td>Odontomachus mayi Mann</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Pachycondyla goeldii (Forel)</td>
<td>8, 9, 10, 11</td>
</tr>
</tbody>
</table>

### Epiphytes for which seed-collecting and/or AG-restriction have been reported

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Ant species observed to collect seeds</th>
<th>Location where AG-restriction determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Araceae</td>
<td>Anthurium gracile Lindl.</td>
<td>A. cf. traili, Ca. femoratus, O. mayi, P. goeldii (6, 9, 10, 11, 15)</td>
<td>Peru, Venezuela (6, 13)</td>
</tr>
<tr>
<td></td>
<td>Anthurium sp.</td>
<td>A. cf. traili, Ca. femoratus (6)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td></td>
<td>Philodendron deflexum Poepp.</td>
<td>A. cf. traili, Ca. femoratus (6)</td>
<td>Peru, Venezuela (6, 13)</td>
</tr>
<tr>
<td>Bromeliaceae</td>
<td>Aechmea longifolia (Rudge)</td>
<td>A. cf. traili, Ca. femoratus (6, 14)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td></td>
<td>A. mertensii Schult.f.</td>
<td>Ca. femoratus; P. goeldii, O. mayi (9, 12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. tillandsioides Baker</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neoregelia eleutheropetala (Ule)</td>
<td>Ca. femoratus (6)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td>Cactaceae</td>
<td>Epiphyllum phyllanthus (L.)</td>
<td>A. cf. traili, Ca. femoratus (6, 15)</td>
<td>Peru (6) but see (14)</td>
</tr>
<tr>
<td>Gesneriaceae</td>
<td>Codonanthe calcarata Hanst.</td>
<td>Ca. femoratus, P. goeldii, unidentified spp (3, 11)</td>
<td>Belize, Venezuela (7, 13)</td>
</tr>
<tr>
<td></td>
<td>C. crassifolia (Focke)</td>
<td>Cr. longispina (2)</td>
<td>Costa Rica (2)</td>
</tr>
<tr>
<td></td>
<td>C. macradenia Donn.Sm.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. uleana Fritsch</td>
<td>A. cf. traili, Ca. femoratus (6, 15)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td>Moraceae</td>
<td>Ficus paraensis Miq.</td>
<td>A. cf. traili, Ca. femoratus (6, 15)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td>Piperaeae</td>
<td>Peperomia macrostachya A.Dietr.</td>
<td>A. cf. traili, Ca. femoratus, unidentified spp (3, 6, 11, 14)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td>Solanaceae</td>
<td>Markea ulei (Dammer)</td>
<td>A. cf. traili, Ca. femoratus (6, 15)</td>
<td>Peru (6)</td>
</tr>
<tr>
<td>Orchidaceae</td>
<td>Coryanthes speciosa (Hook.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epidendrum imatophyllum Lindl.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1 Continued

<table>
<thead>
<tr>
<th>Family</th>
<th>Species Name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Araceae</td>
<td><em>Philodendron myrmecophilum</em> Engl.</td>
<td>(1, 4, 5, 13)</td>
</tr>
<tr>
<td>Bromeliaceae</td>
<td><em>Streptocalyx angustifolius</em> Mez</td>
<td>(1, 11)</td>
</tr>
<tr>
<td>Moraceae</td>
<td><em>Ficus myrmecophila</em> Warb</td>
<td>(1, 4, 5)</td>
</tr>
<tr>
<td>Solanaceae</td>
<td><em>Markea formicarum</em> Dammer</td>
<td>(1, 11, 12)</td>
</tr>
<tr>
<td>Clusiaceae</td>
<td><em>Clusia</em> sp. (5, 13, 16)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Occurrence of nine volatile compounds in AG seeds; letters indicate that the compound was detected in at least one seed sample of a given species by Seidel et al. (1990) (a) or Youngsteadt et al. unpublished data (b). *A. ernestii* and *N. eleutheropetala* were not examined in (b).

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthurium gracile</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Anthurium ernestii</td>
<td>a</td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Philodendron megalophyllum</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Neoregelia eleutheropetala</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Aechmea longifolia</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Epiphyllum phyllanthus</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Codonanthe uleana</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Ficus paraensis</td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Peperomia macrostachya</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Markea ulei</td>
<td>a,b</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

Compounds: 1, 6-MMS (methyl 2-hydroxy-6-methylbenzoate); 2, benzothiazole; 3, vanillin (4-hydroxy-3-methoxybenzaldehyde); 4, 1-(2-hydroxy-6-methylphenyl)ethanone; 5, 1-(2,4-dihydroxyphenyl)ethanone; 6, geranyl linalool ((6E,10E)-3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol); 7, β-springene ((6E,10E)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene); 8, geranylgeraniol ((3E,6E,10E)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene); 9, α-springene ((3E, 6E, 10E)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Hostile toward allocolonial conspecifics</th>
<th>Hostile toward allocolonial partner species</th>
<th>Cuticular chemistry acquired from partner species</th>
<th>Parabiosis obligate</th>
<th>Polydomous</th>
<th>Polygynous</th>
<th>Subfamily*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 5, 6, 7, 8, 13, 15</td>
<td>no, or only slightly</td>
<td>occasionally (whole-nest assay only)</td>
<td>variable</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>F</td>
</tr>
<tr>
<td>7, 8, 12</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>7, 8, 12</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>1, 3, 5</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 3. Summary of characteristics of parabiotic ants.
<p>| | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Camponotus rufifemur</strong></td>
<td>F</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td>unclear; compounds are shared</td>
<td></td>
<td>only toward some colonies</td>
</tr>
<tr>
<td><strong>Crematogaster modiglianii</strong></td>
<td>M</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td>unclear; compounds are shared</td>
<td></td>
<td>yes</td>
</tr>
<tr>
<td><strong>Crematogaster spKfmA240</strong></td>
<td>F</td>
<td>rarely</td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td>?</td>
<td></td>
<td>?</td>
</tr>
</tbody>
</table>


* D = Dolichoderinae; F = Formicinae; M = Myrmicinae; P = Ponerinae

† The description of C. levior (Longino, 2003) includes obligate parabiosis as a defining characteristic, but it is reported as a free-living species in French Guiana (Vantaux et al. 2007 and references therein).
Figure 1. Structures of compounds found in AG seeds (see Table 1).
Species-Specific Seed Dispersal in an Obligate Ant-Plant Mutualism

This chapter was prepared for submission to PLoS ONE with coauthors Jeniffer Alvarez Baca¹, Jason Osborne² and Coby Schal³.

1 Facultad de Ciencias Biológicas, Universidad Nacional de San Antonio Abad del Cusco, Av. de la Cultura, Nro. 733, Cusco, Perú, 2 Department of Statistics, North Carolina State University, Box 8203, Raleigh, North Carolina, United States of America 3 Department of Entomology and W. M. Keck Center for Behavioral Biology, North Carolina State University, Box 7613, Raleigh, North Carolina, United States of America.
Abstract

Throughout lowland Amazonia, arboreal ants collect seeds of specific plants and cultivate them in nutrient-rich nests, forming diverse but species-specific, obligate symbioses called Neotropical ant-gardens (AGs). The ants depend on their symbiotic plants for nest stability, and the plants depend on AGs for substrate and nutrients. Although the AGs are limited to specific participants, it is unknown at what stage specificity arises, and seed fate pathways in AG epiphytes are undocumented. Here we examine the specificity of the ant-seed interaction by comparing the ant community observed at general food baits to ants attracted to and removing seeds of the AG plant *Peperomia macrostachya*. We also compare seed removal rates under treatments that excluded vertebrates, arthropods, or both. In the bait study, only three of 70 ant species collected *P. macrostachya* seeds, and 84% of observed seed removal by ants was attributed to the AG ant *Camponotus femoratus*. Arthropod exclusion significantly reduced seed removal rates, but vertebrate exclusion did not. We provide the most extensive empirical evidence of species specificity in the AG mutualism and begin to quantify factors that affect seed fate in order to understand conditions that favor its departure from the typical diffuse model of plant-animal mutualism.
Introduction

To survive, seeds must arrive at suitable germination sites. This poses special problems for epiphyte seeds, which must move against gravity to arrive at very specific and patchy germination sites. The vast majority of epiphytes meet these requirements by producing abundant wind-dispersed diaspores or by attracting vertebrate frugivores likely to deposit seeds in feces on branches above the ground [1,2]. A small but conspicuous minority of epiphytes rely upon ants for dispersal. Throughout the Amazon basin, this strategy is represented by some 15 epiphyte species that grow exclusively or principally in arboreal carton nests built by ants, forming abundant hanging gardens known as ant-gardens (AGs) (Fig. 1) [3-6]. In this habitat, epiphytes are limited by substrate and nutrient availability, and AGs are considered the most important substrate for vascular epiphytes due to their porous texture and enriched N, K, and P relative to other insect carton or surrounding soil [7,8]. AG epiphytes further rely upon ants for defense against herbivores and seed dispersal [5,9,10].

Ant-gardens are notable not only as the product of an unusual seed dispersal strategy in epiphytes, but as the most complex form of ant-plant symbiosis [11]. AGs are initiated when ants collect seeds of specific epiphytes and carry them to their nests, incorporating them into the carton walls [3,5,12]. AG ants collect the seeds in response to chemical cues and independently of nutritional rewards, removing them directly from the plants, from vertebrate feces and from the soil surface [3,5,13,14]. The ants rely upon the roots and leaves of the germinated plants for nest structure and dehumidification; without epiphytes,
the carton nests disintegrate during the rainy season [15]. The AG mutualism also makes it possible for the ants to colonize resource rich microhabitats independently of pre-existing nest substrates, an advantage that may have led to the dominance of AG ants in lowland Amazonia [5,16]. In southeastern Perú, AG territories occupied 16% to 39% of a 12 km transect, depending on habitat type [5]. Further, in those same forests AG ants are the most frequently encountered, numerically abundant and behaviorally dominant species in arboreal ant samples and at terrestrial baits [5,16].

The AG flora and fauna are taxonomically diverse, but specific and consistent through time and space. AG-restricted epiphytes occur in seven different plant families, and AG construction has been confirmed in four ant species in three subfamilies, all of which represent independent origins of traits necessary for the AG symbiosis [5,6,17]. Although the AG interaction involves more than two partners, its specificity is nonetheless in contrast to the diffuse nature of most plant-animal interactions, such as seed dispersal and many pollination mutualisms, that inform current understanding of mutualism [18-21, but see 22]. It is therefore of interest to elucidate the mechanisms that favor and maintain the pattern of specificity in this seed-dispersal mutualism.

Some AG seeds bear adhering fruit pulp, oils, or lipid-rich elaiosomes, which could motivate seed collection by multiple ant species, but published observations support some level of specialization in the ant-seed interaction. Orivel and Dejean [3] demonstrated that the AG ants *Camponotus femoratus* (Fabricius) and *Pachycondyla goeldii* (Forel) collect seeds of AG epiphytes even when fruit pulp and elaiosomes have been removed. *C.*
**femoratus** did not carry seeds of non-AG congeners of AG plants [5, EY pers. obs.]. On the other hand, Davidson [5] presented seeds of three species of AG plants, with putative food rewards intact, to single colonies of four generalist non-AG ant species; three of those ant species (*Camponotus sericeiventris* (Guirin-Mineville), *Dolichoderus attelaboides* (Fabricius) and *Cephalotes spinosus* (Mayr)) did not carry AG seeds, while a fourth (*Dolichoderus bidens* (Linnaeus)) did. Thus 25% of non-AG ants were observed to carry the AG seeds, but it is unclear, based upon this small sample size of ant species and colonies, what degree of specificity would be expected in the ant community at large. In addition to the role of ants in AG seed dispersal, many other possible influences on AG seed fate are unknown.

We can conceive of three explanations for the AG-restricted distribution of AG epiphytes: (1) only AG ants are attracted to and collect AG seeds; (2) other ants are attracted to AG seeds but are excluded from collecting them by the abundant and dominant AG ants; and (3) other organisms such as mammals or non-AG ants also collect the seeds but destroy them or deposit them in locations unsuitable for plant survival. Here, we distinguish among these alternatives by comparing the community of ants that could potentially interact with seeds (i.e., ground-foraging species detected at general food baits) to those actually visiting and removing seeds of the abundant AG plant *Peperomia macrostachya* (Vahl). We further address factors that influence dispersal and predation of *P. macrostachya* seeds by comparing seed removal rates under selective exclusion of vertebrates, arthropods, neither,
or both. Finally, we present an estimate of seed survival for those seeds that are finally retrieved to AG carton.

Results

Bait study

Of ant species that could have potentially interacted with AG seeds, very few actually did so. Ants were observed at 105 (97%) of the 108 terrestrial bait stations when baited with food and only at 28 (26%) of the stations when baited with *P. macrostachya* seeds. At 20 of these 28 stations (71%), seeds were visited only by the AG ant *C. femoratus,* or *C. femoratus* together with its heterospecific nestmate *Crematogaster levior* Longino. Although *C. femoratus* was also the single most common visitor to food baits, it accounted for a much lower proportion of visited food baits than seed baits: 26 of 105 stations (25%). Seventy ant species were collected overall: 68 at food baits and eight at AG seed baits. Most baits hosted one species at a time, with a maximum of five species collected during a single observation. Only three of the eight species at AG seeds were observed to collect the seeds (Table 1), and multiple workers of all three species removed seeds in an apparently "purposeful" manner, grasping seeds from the tray and walking quickly away with them, continuing to carry them until disappearing into leaf litter or dense vegetation where we did not follow. The conditional probability of *C. femoratus* appearing at seeds given its presence at a bait station differed significantly from the same conditional probability of non-AG ants
\( \chi^2 = 31.3, \text{ df } = 1, P < 0.0001 \). The odds ratio for these conditional probabilities was 
\((21/9)/(18/86) = 11.1, \) with a 95% CI of 4.4 to 28.3. In other words, when present at a bait 
station, \textit{C. femoratus} was 11.1 times more likely than other ants to appear at the seed bait.

Of 2205 seeds presented at bait stations, 43\% (938 seeds) disappeared during the 
course of the observations. Ants accounted for 42\% of seeds removed (390 seeds). \textit{C. femoratus} was responsible of the overwhelming majority of ant-removal of AG seeds (Fig. 2). \textit{Sericomyrmex} sp. 1 (determined to genus by T. Schultz) and \textit{Camponotus} sp. 2. (determined by W. Mackay as an undescribed species) were also observed to carry seeds (Fig. 2). (Though unobserved ants may have removed some seeds, we restrict these results and subsequent discussion only to observed interactions.) Cockroaches and crickets were 
often found at baits, and seeds sometimes clung to their legs or were dislodged from the tray.

Ants that occurred at seed baits without collecting seeds engaged in various 
behaviors, none of which appeared to be direct use of seeds. \textit{C. levior} occurred at seed baits 
only with \textit{C. femoratus}, with which it shares nests and foraging trails. Although \textit{C. levior} 
was observed foraging alone (independently of \textit{C. femoratus}) at food baits, it never appeared 
to forage independently for seeds, and made no visible attempt to remove seeds. 
\textit{Crematogaster brasiliensis} Mayr, a common species at food baits, appeared at only one seed 
bait and did not interact directly with seeds but appeared to investigate the plastic tray itself 
rather than the seeds. \textit{Camponotus sericeiventris} (Guérin-Méneville) was represented at seed 
baits by a single individual which repeatedly antennated both plate and seeds and displayed 
alarm-like behavior. \textit{Dolichoderus imitator} Emery and \textit{Sericomyrmex} sp. 2 were also
represented by single individuals, apparently exploring. *Dolichoderus bispinosus* (Olivier) appeared at two seed baits where it investigated seeds; four seeds disappeared from one bait where this species was found during the morning observation, but no further seeds were removed during 20 min of direct observation or when the bait was checked again 30 min after that. By the afternoon observation, *D. bispinosus* had been replaced at the bait by *C. femoratus*.

**Exclusion experiment**

Arthropod exclusion (Tanglefoot) (Fig. 3) significantly decreased the number of *P. macrostachya* seeds removed from the seed plates (Fig. 4, Table 2). The effect of *C. femoratus* on seed removal was significant only in the absence of Tanglefoot; where AG ants were present, many more seeds were removed from Tanglefoot-free plates than Tanglefoot-treated plates (Fig. 4). In AG territories, many *C. femoratus* became trapped in Tanglefoot, which had to be cleaned often to prevent foragers reaching seeds by walking on trapped ants. In non-AG territories, Tanglefoot occasionally trapped an apparently idiosyncratic variety of insects but not ants.

Vertebrate exclusion (wire mesh cages) had no significant effect on seed removal, although cages tended to diminish seed removal in non-AG territories. A total of 34 undispersed seeds appeared to have been chewed or crushed and left on the plates. Twenty nine of these were left at two non-AG plots, and five in a single AG plot. The unknown culprit(s) accessed seeds in all exclusion treatments without disturbing cage placement, and is likely to be an arthropod capable of jumping and/or flying.
Seed fate in AG carton

We observed a total of 794 *P. macrostachya* plants in 10 AGs. Of these, 91% (720 plants) were recently germinated seedlings, 2% (18) were juvenile and 7% (56) were mature plants. Given our three assumptions (see Methods), this census yields a maximum seedling-to-adult transition probability of 8%.

Discussion

We present the strongest available evidence that the AG ant-seed interaction is highly specific in lowland forest of the Peruvian Amazon, and thus represents an exception to the general understanding of seed-dispersal mutualisms as generalized and diffuse interactions [18,21,23,24]. Ants that removed *P. macrostachya* seeds were a very small subset of the generalist ground-foraging fauna, and the AG ant *C. femoratus* was by far the most abundant and persistent remover, and thus disperser, of AG seeds. Common granivorous species were conspicuously absent. We therefore reject the hypothesis that competition between ant species or post-dispersal events limit *P. macrostachya* seeds to AGs. Instead, the distribution of *P. macrostachya* in AG ant nests arises largely due to specificity of the ant-seed interaction, and probably also due to seed or seedling death when removed by other species.
Factors affecting *P. macrostachya* seed fate

The actual and putative seed fate pathways we propose for *P. macrostachya* are represented graphically in Fig. 5. These pathways include seed removal by *C. femoratus*, other ants, and vertebrates. The few removal events attributable to ants other than *C. femoratus* (less than 3% of all seeds presented at baits) were unlikely to result in successful germination. The natural history of *Camponotus* sp. 2 (Table 1) is unknown; it occurred once at a food bait and twice at seed baits, each time at night in or near bamboo thickets. *Sericomyrmex* and other lower attine species have been previously reported as important secondary dispersers of seeds in various tropical habitats, where they retrieve typically vertebrate-dispersed seeds with adhering fruit. The cleaned seeds are either retained in fungus gardens or later discarded in viable condition in rubbish heaps [25,26]. It is unclear whether the *Sericomyrmex* observed in this study would keep *P. macrostachya* seeds in the nest for fungus-culturing, or discard them. In either case, because *Sericomyrmex* nests on the ground and AG species succeed only in the canopy, the seeds would be doomed or would await further dispersal. All instances of non-AG ants removing *P. macrostachya* seeds took place at bait stations where *C. femoratus* did not occur. Nevertheless, casual observations of *Pheidole astur* Wilson retrieving seeds near *C. femoratus* foraging trails indicates that it is not impossible for other species to collect the seeds even within AG territories (Fig. 5). The non-AG ant *Dolichoderus bidens* has also been observed to carry AG seeds, including *P. macrostachya*, when directly confronted with the seeds [5], but *D. bidens* was not observed in the present study.
Both the bait study and the exclusion experiment support our assertion that *C. femoratus* is the main disperser of AG seeds in the study area. The major effect of Tanglefoot both within and away from AG territories further suggests that the unobserved seed removers in both experiments are likely to be arthropods rather than vertebrates. The absence of a vertebrate effect is unusual but not unprecedented with small tropical seeds [27-29]. A vertebrate effect could have been masked by the experiment itself if small vertebrates were deterred by the presence of plastic plates and/or Tanglefoot. Our experiments do not address the possibility of vertebrates removing seeds directly from the AG plants. This phenomenon has rarely been observed, but Davidson [5] noted *P. macrostachya* seeds in bat droppings and reported both birds and monkeys feeding on fruits of other AG epiphytes. Sticky *P. macrostachya* seeds may also be dispersed by adhering to vertebrates that visit AGs to consume other fruits [13]. *C. femoratus* likely removes AG seeds from vertebrate feces, so vertebrate consumption does not categorically doom a seed, and may represent one of few opportunities for long distance dispersal in a system where ants usually return seeds to the garden of origin or to a neighboring garden of the same ant colony [5]. Occasional dispersal to suitable sites by arboreal vertebrates may also have led to the establishment of the few *P. macrostachya* plants observed to grow independently of AGs. Finally, there is some evidence that ants may later build carton nests around AG plants that establish independently [30, G. Mathieu personal communication]. This phenomenon has yet to be thoroughly documented, and has not been observed in Perú (E.Y. personal observation, D. Davidson, personal communication) but deserves further attention, and
could be critical to *P. macrostachya* fate and distribution in some regions. There may be a role for all these pathways in determining AG seed fate, but their importance requires further investigation (Fig. 5).

In the exclusion experiment, 88% of arthropod-accessible *P. macrostachya* seeds presented in AG territories were removed from the plates. In the bait study, we observed a lower overall level of seed removal in AG territories: 65% of seeds were removed from bait stations where *C. femoratus* was observed at least once at either food or seeds. The discrepancy is probably due to at least two differences in seed presentation between the two studies. In the exclusion experiment, seeds were intentionally located centrally in AG foraging territories, and were present for four consecutive days. In the bait study, stations were located without respect to AG territories and seeds were present for only one day, so that some bait stations were peripheral to AG territories, visited by few ants, and/or discovered only toward the end of the observation period. In both experiments, seed removal was much lower in the absence of AG ants: 52% and 31%, respectively.

When seeds do arrive in AG carton, the probability of survival is still low. Ants incorporate hundreds of seeds into the carton of even a single nest, which can only support a few adult plants. Even in a single snapshot census, these seedlings are almost 13 times more abundant than mature plants on gardens; it is likely that some retrieved seeds do fail to germinate, and that there is some seedling turnover on the nest during the *P. macrostachya* fruiting season. Therefore, our estimate of seedling survival is probably an upper bound, and mortality must approach 100% as seedlings are winnowed and few adult plants establish.
Nevertheless, the estimated survival rate of $\leq 8\%$ is comparable to seed or seedling survival rates measured in other ant-dispersed seeds and other epiphytes: survival rates of either seeds that have already arrived at ant nests or potentially suitable branches, or of young seedlings that have just germinated in such locations, can range from about 3\% to 30\% [e.g. 31-35]. For AG plants, establishment opportunities may occur mainly when carton is added to existing nests or when new nests are initiated.

Nevertheless, many seed fate pathways remain unexplored in the AG system. Though they did not appear in the present study, other ant species are known to carry AG seeds and build gardens. *Azteca* spp. sometimes carry *P. macrostachya* seeds [5], while the gardening species *Pachycondyla goeldii* and *Odontomachus mayi* rarely carry or cultivate *P. macrostachya*, instead demonstrating strong preference for other AG plants [3]. The relative importance of these other gardening species to seed fate in *P. macrostachya* and other AG seed species remains to be determined. Future work should also investigate agents of long-distance dispersal and their contribution to gene flow in AG plants, compare patterns of seed movement among the taxonomically diverse AG epiphytes, and assess factors affecting seed fate over a wider geographic range.

**Why don't other ants collect *P. macrostachya* seeds?**

Further evidence of species-specificity in the *P. macrostachya-C. femoratus* interaction comes not from direct observation of seed movement, but from the conspicuous absence of ants that might ordinarily collect seeds. For instance, the extremely diverse genus *Pheidole* includes many granivorous species and, to our knowledge, is reported at seeds in
every systematic study of small-seed dispersal and granivory in the New World tropics [26-28,36-40]. Among the Pheidole species collected at food baits in the present study, at least P. fimbriata, P. nitella and P. peruviana are known or suspected granivores [40]. P. astur occasionally collected P. macrostachya seeds when these were presented to C. femoratus in behavioral assays for a different experiment. Although P. astur did exploit food in the bait study, it was not observed at seed baits so its retrieval of AG seeds could not be quantified. The absence of the genus Pheidole at P. macrostachya seed baits, despite its detection at 36 general food baits, suggests that these seeds may repel or deter at least some ant species.

In addition to the scarcity of granivores at seed baits, comparison with other tropical ant-seed interaction studies suggests that P. macrostachya seeds are exceptionally under-visited by ants. In a study of ants using six different nonmyrmecochorous seed species in Brazil, Pizo and Oliveira [28] found that 90% of the surveyed seeds were attended by ants at least once during six surveys in a 24 hour period even though these seeds did not offer specialized ant rewards. By comparison, in the present study only 26% of P. macrostachya seed baits were attended by ants during three surveys in the same time period, and only 17% by ants other than C. femoratus.

Seed size can inform which ant species utilize available seeds [26,28]. It seems unlikely, however, that seed size is wholly responsible for the patterns observed in this study. At least half the ant species collected at food baits were clearly large enough that individual workers could have easily carried P. macrostachya seeds. Though other studies have found that C. levior attempts to carry AG seeds, but is unable to do so because of its small size or
because it is displaced by *C. femoratus* [3,5], we were unable to confirm or refute these observations in the present study. *C. levior* was often present at seed baits together with *C. femoratus*, but *C. levior* never foraged independently for seeds as it did for food, nor did it make visible attempts to carry seeds. Thus it is unclear whether *C. levior* arrived at *P. macrostachya* seed baits in this study because it is attracted to them, or whether its presence was an incidental result of shared trail use with *C. femoratus*.

Furthermore, if the seeds were attractive to other species, ants of any size should still have been observed interacting with the seeds even if not removing them. Instead, when we did find non-AG ants at AG seed baits, they appeared to ignore the seeds, or in the case of *C. sericeiventris*, to be alarmed by them—an outcome that has also been reported previously [5].

*P. macrostachya* seeds emit many phenolic and terpenoid volatiles, and the component geranyl linalool is shared among at least eight AG seed species [14, E.Y. unpublished data]. These components, though accepted by and even attractive to *C. femoratus*, could act as deterrents to other species; geranyl linalool in particular is toxic to many ants [41]. A related phenomenon occurs in flowering plants that produce ant-repellent floral scents [42], nectar [43], or pollen [44] that prevent detrimental activities of ants on flowers—namely, thieving nectar and deterring pollinators. Floral repellents may be particularly well developed in plants that are adapted to attract or house ant-guards [45], and there is evidence that, as we suggest for *P. macrostachya*, such floral repellents can be widely effective ant deterrents while still admitting one or a few ant species [46].
To confirm repellency of *P. macrostachya* seeds to non-AG ants, it would be interesting to conduct additional experiments comparing ants at AG seeds to ants utilizing alternative seed baits. We did not undertake comparison to other seeds in the present study because the a priori choice of alternative bait would have been problematic. The present results suggest that generalists, predators and granivores are all underrepresented at *P. macrostachya* seeds, and these observations could be further tested by comparison to seeds that are known to attract such ants in other habitats—namely, lipid-rich, fruity or elaiosome-bearing seeds for generalists and predators [28,47], and dry seeds such as barley for granivores [26,48]. Seed extracts could also be tested for repellency to non-AG ants in an olfactometer assay [14,41].

Other ant species reported as gardeners (*Odontomachus mayi*, *Pachycondyla goeldii*, and *Azteca* spp.) were not detected in the present study. A single *Pachycondyla* garden has been noted at the study site, and *Azteca* gardens, although they do host *P. macrostachya* plants, account for no more than 5% of gardens in terraza and bajío habitats at the site. *Azteca* species did not appear at food or seed baits in this study.

**Why specialize?**

Overall, we describe an unusually specific and intimate seed-dispersal mutualism and provide the first empirical account of seed movements in an ant-garden epiphyte. Although the AG system is a case of interacting guilds rather than a one-to-one partnership—some 15 epiphyte species grow in gardens built by four ant species over the range of the interaction—the mutualism nonetheless contrasts with the current
understanding of seed dispersal as a general interaction in which animal and plant partners interact in diffuse and asymmetrically dependent networks. Herrera [24] described factors that should limit specialization in seed-dispersal mutualisms, including unpredictability of germination sites in space and time, and weak reciprocal selective pressure by plants and dispersers. AGs, however, make suitable germination sites predictable. At the study site, *C. femoratus* appears to be the only ant capable of dispersing the seeds to suitable sites, and removal by other means will nearly always have negative consequences. It is also noteworthy that AG partners remain associated after the act of dispersal, throughout their life histories, and both plants and ants depend upon this intimate cohabitation for survival. They may therefore exert stronger and more consistent selective pressures upon one another than free-living mutualists.

This study, however, provides only a snapshot of the interaction in time and space. To clarify the selection pressures that promote or prevent coevolution in AG partners, future studies should compare the benefits (nutrients, protection, seed dispersal efficiency) conferred by different AG ant species that occur in other regions, and the seed traits to which those ants respond. For example, Youngsteadt et al. [14] identified a blend of volatile compounds from *P. macrostachya* seeds that attracted *C. femoratus*, and chromatographic fractions of *P. macrostachya* extract that elicited seed-carrying behavior. It is not known whether these same seed characteristics are responsible for the behavior of all AG ants, or whether different species may exert conflicting selective pressures upon the seeds. Similarly, AG ants interact with multiple plant partners. Whether the specificity and selective pressures
in the *C. femoratus-P. macrostachya* mutualism are duplicated in all AG ant-seed interactions remains to be determined.

**Materials and Methods**

**Field site and study species**

Studies were conducted during October through December, 2006, at the Centro de Investigación y Capacitación Río Los Amigos in Madre de Dios, Perú (located at 12º34'07"S, 70º05'57"W) consisting of floodplain forest (bajío), upland forest (terraza) and bamboo thickets (pacal). AGs constructed by the ant *Camponotus femoratus* are abundant in both the bajío and terraza habitats, with aggregations of 2–30 nests occurring along trails in those habitats at an average interval of about 300 m (E.Y. unpublished data). *C. femoratus* occupied more than 95% of AGs in these habitats (*n* = 168 AGs censused), and 98% of *C. femoratus* nests also housed the parabiotic ant *Crematogaster levior*. The other AGs at the site were constructed by *Azteca* species. Nine species of epiphytes regularly occur in AGs at the field site; most of the 162 *C. femoratus* gardens surveyed hosted a single plant species and 44% hosted two or more plant species, occasionally up to six or seven (E.Y. unpublished data). The most abundant is *Peperomia macrostachya*, which occupies 91% of gardens at the site and which Davidson [5] described as an AG pioneer species, among the first to grow in newly established gardens. This species is considered AG-restricted, rarely occurring outside of ant nests; of 674 *P. macrostachya* plants observed at a nearby site [5],
only five individuals grew independently of AGs. We therefore assumed that *P. macrostachya* was a representative AG plant central to the AG mutualism, and used freshly collected mature *P. macrostachya* seeds in all seed removal experiments described below.

All seeds were collected with forceps and transported in clean Petri dishes.

**Bait study**

We surveyed the ant assemblage at 108 sampling stations placed every 25 or 50 m along sections of the established trail system, 1 m off the trail and randomly assigned to the left or right of the trail. Of the 108 stations, 27 were in bajío habitat, 69 in terraza, and 12 in pacal. Each sampling station was in place for two days and was baited one day with 15 *P. macrostachya* seeds, the other day with tunafish and strawberry jam. Protein and sugar baits are common and reproducible means of assessing overall ant diversity at a site [48] and we expected tuna and jam to attract potential seed predators as well as potential dispersers, which are often generalist or even predatory ants [47,49]. The order of bait presentation was randomized. Baits were presented on 4.3 cm² perforated plastic trays held in place with wire anchors. Baits were first set out in the morning about 0700 hours and replenished throughout the experiment. Ants were observed and collected at the baits three times throughout the day over the course of 12–14 hours: once in the morning (by 0900), once in the afternoon (between 1300 and 1600), and once after dark (between 1930 and 2100, using red-filtered light). At each seed bait, number of seeds removed since the previous visit was noted. Where seeds had been removed, or where ants were present at seed baits, the sampling station was observed for 10–20 min and re-visited again about 30 min later. Ants were
sorted and identified to species or morphospecies, and specimens are deposited at the Universidad Nacional de San Antonio Abad del Cusco in Cusco, Perú. We tabulated a 2x2 contingency table in which ants were categorized as either *C. femoratus* or not *C. femoratus*. For each of these two classes, we counted the number of bait stations at which ants visited seeds, and the number of stations at which they visited food but not seeds. We used the FREQ procedure in SAS version 9.3 to perform a chi-square test that compared the conditional probability of *C. femoratus* appearing at seeds given its presence at a bait station (food or seeds) to the same conditional probability for the class of all other ants combined.

**Exclusion experiment**

To further examine factors affecting dispersal and predation of *P. macrostachya* seeds, we conducted an ant and vertebrate exclusion experiment. Trials were conducted in a randomized complete block split plot design with four blocks, two plots per block, and four treatments per plot. The two plots in a block were in the same habitat type and were monitored on the same days, but one was within *C. femoratus* foraging territory (as previously determined by *C. femoratus* presence at food and seed baits) and one was not. Plots were 1 m², and each included four treatments positioned at the four corners of the plot: exclusion of both vertebrates and ants with wire mesh cages and Tanglefoot (Tanglefoot Co., Grand Rapids, MI); exclusion of vertebrates with cages only; exclusion of arthropods with Tanglefoot only; and no exclusion (Fig. 3). For each treatment, 15 seeds were placed in a 4.3 cm² perforated plastic tray that was glued in the center of a 13 cm diameter perforated plastic plate and secured to the forest floor with wire anchors. To exclude walking
arthropods, Tanglefoot was spread in a 3–4 cm band around the plate perimeter. For vertebrate exclusion, cages (15 cm square, 7.5 cm high, made of 1.5 cm wire mesh) were secured over the plates with wire anchors. Initial placement of the four treatments within a plot was randomized. Twenty four hours later, seeds were counted and replaced, and the positions of treatments were rotated so that over the course of four days, a total of 60 seeds were subjected to each treatment, and each treatment experienced each position within the plot.

The number of seeds removed from each treatment in each plot was summed over the four days. To test for effects of *C. femoratus* and exclusion of vertebrates or arthropods on seed removal, seed counts were first subjected to the empirical logistic transformation to achieve homogeneity of variance [50]. The MIXED procedure in SAS 9.1.3 was used to fit a mixed model with fixed effects for the whole plot factor AG ants and the split-plot factors Tanglefoot and cage, and random effects of block and block x AG ant interaction. Because there was a significant interaction between Tanglefoot and AG ants, the simple effect of AG ants was tested separately in the presence and absence of Tanglefoot. The Satterthwaite option was used within the MIXED procedure because *F* -ratios for these simple effects were constructed using error terms that were linear combinations of multiple mean squares from the ANOVA table [51].

**Seed fate in AG carton**

To estimate the survival success of seeds retrieved to AG carton, we censused *P. macrostachya* plants in 10 AGs occupied by *C. femoratus*, scoring individuals as seedlings
(cotyledons only), established juvenile plants (mature leaves but no reproductive structures),
or adult plants (reproductive structures present). Censuses were conducted in December
2006, near the end of *P. macrostachya* fruiting season, which lasts 2–3 months in the late
dry season and early rainy season [52, E. Y. personal observation]. We censused gardens of
which we had an unobstructed view, or which had recently fallen to an accessible height.
Each garden was censused once. To estimate seed survival based on these data, we made
three assumptions, supported by the following observations. First, *P. macrostachya* seeds
have a very high germination rate once they contact a moist substrate, even if that substrate
is inappropriate. We have observed them to germinate within a few days on the ground
beneath AGs, on seed trays left out after the conclusion of experiments, and in AG carton
samples kept in a plastic box with or without ants. We therefore assumed that all seeds
retrieved to an AG would sprout to the seedling stage. Second, we have not found seed
 caches within gardens despite opening many nests. We therefore assume that all seedlings
on an AG represent seeds collected during the same fruiting season. Finally, we assume that
visible seedlings represented the sum of the season’s seed-collecting, i.e., that seedlings had
100% survival during the months of *P. macrostachya* fruiting that led up to the census.
While highly speculative at this point, these assumptions provide the foundation for the only
available estimate of seed success in the AG system, and all assumptions are designed to
give an upper bound to the possible range of seed survival rates in *P. macrostachya*. Given
these assumptions, we used the observed snapshot ratio of adult plants to seedlings to
estimate the maximum probability that a seed retrieved to an AG matures to an adult plant.
Acknowledgments

We thank Silvia Castro and Erick Yabar for their assistance. John Lattke, John Longino, William Mackay, Amy Mertl, Ted Schultz, James Trager, Philip Ward and Alex Wild identified ant specimens. We thank Rob Dunn, Jules Silverman and Ed Vargo for critical comments on a draft of this manuscript. Permission to work in the Los Amigos conservation concession was granted by the Instituto Nacional de Recursos Naturales (INRENA) of Perú.

References


Table 1. Ant species recorded at general food baits and at ant-garden seed baits on transects in southeast Perú

<table>
<thead>
<tr>
<th>Classification</th>
<th>Number of food baits visited</th>
<th>Number of seed baits visited</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ponerinae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Ectatomma tuberculatum</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2 E. lugens</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3 Gnamptogenys sp. (striatula group)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4 Gnamptogenys moelleri</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5 Odontomachus laticeps</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6 Pachycondyla constricta</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7 P. crassinoda</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8 P. harpax</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>9 Paraponera clavata</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Myrmicinae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Apterostigma auriculatum</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11 Apterostigma sp. (pilosum group)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12 Crematogaster brasiliensis</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>13 C. levior</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>14 C. limata</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>15 C. sotobosque</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>16 C. tenuicula</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>17 Megalomyrmex balzani</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>18 Ochetomyrmex neopolitus</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>19 O. semipolitus</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>20 Pheidole astur</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>21 P. biconstricta</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>22 P. deima</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>23 P. embolopyx</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>24 P. fimbriata</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>25 P. laidlowi</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>26 P. nitella</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>27 P. scolioceps</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>28 P. xanthogaster</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>29 Pheidole sp. (nr. deima)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>30 Pheidole sp. (nr. leptina)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>31 Pheidole sp. (nr. peruviana)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>32 Pheidole sp. 1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>33 Pheidole sp. 2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>34 Pheidole sp. 3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>35 Pheidole sp. 4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>36 Pheidole sp. 5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>37 Pheidole sp. 6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>38 Pheidole sp. 7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>39 Pheidole sp. 8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>40 Pheidole sp. 9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>41 Pheidole sp. 10</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table 1 Continued

<table>
<thead>
<tr>
<th>Ant Species</th>
<th>42 Sericomyrmex sp. 1</th>
<th>43 Sericomyrmex sp. 2</th>
<th>44 Solenopsis virulens</th>
<th>45 Solenopsis-2</th>
<th>46 Solenopsis-3</th>
<th>47 Trachymyrmex farinosus</th>
<th>48 Trachymyrmex cf. bugnioni</th>
<th>49 Trachymyrmex sp.</th>
<th>50 Wasmannia auropunctata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Formicinae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51 Brachymyrmex cf. longicornis</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52 Camponotus indianus</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53 C. amoris</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54 C. atriceps</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55 C. cacicus</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56 <em>C. femoratus</em></td>
<td></td>
<td></td>
<td>26</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57 <em>C. lespesi</em></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58 C. novogranadensis</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59 C. sericeiventris</td>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Camponotus sp. cf. cressoni</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61 Camponotus sp. 1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62 <em>Camponotus sp. 2</em></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 Camponotus sp. 3</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64 Paratrechina sp. 1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65 Paratrechina sp. 2</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolichoderinae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66 Dolichoderus attelaboides</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67 Dolichoderus imitator</td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68 Dolichoderus bispinosus</td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomyrmicinae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69 Pseudomyrmex tenuis</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 Pseudomyrmex sp. cf. tenuis</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Food baits consisted of canned tunafish and strawberry jam; seed baits were *P. macrostachya* seeds.

Ant species that collected *P. macrostachya* seeds are in bold type.
Table 2. Results of ANOVA testing for fixed effects of cage, Tanglefoot and AG ants in the exclusion experiment

<table>
<thead>
<tr>
<th>Source</th>
<th>Numerator DF</th>
<th>Denominator DF</th>
<th>Sum of squares</th>
<th>F-ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage</td>
<td>1</td>
<td>18</td>
<td>0.99</td>
<td>0.46</td>
<td>0.506</td>
</tr>
<tr>
<td>Tanglefoot</td>
<td>1</td>
<td>18</td>
<td>168.87</td>
<td>78.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AG ants</td>
<td>1</td>
<td>3</td>
<td>24.60</td>
<td>5.28</td>
<td>0.105</td>
</tr>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage x Tanglefoot</td>
<td>1</td>
<td>18</td>
<td>0.99</td>
<td>0.46</td>
<td>0.505</td>
</tr>
<tr>
<td>Cage x AG ants</td>
<td>1</td>
<td>18</td>
<td>3.23</td>
<td>1.50</td>
<td>0.105</td>
</tr>
<tr>
<td>Tanglefoot x AG ants</td>
<td>1</td>
<td>18</td>
<td>49.94</td>
<td>23.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Simple effects of AG ants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG ants, Tanglefoot absent</td>
<td>1</td>
<td>5.15</td>
<td>72.32</td>
<td>18.21</td>
<td>0.006</td>
</tr>
<tr>
<td>AG ants, Tanglefoot present</td>
<td>1</td>
<td>5.15</td>
<td>2.22</td>
<td>0.56</td>
<td>0.485</td>
</tr>
<tr>
<td>Cage x Tanglefoot x AG ants</td>
<td>1</td>
<td>18</td>
<td>0.08</td>
<td>0.04</td>
<td>0.846</td>
</tr>
<tr>
<td><strong>Random effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocks</td>
<td>3</td>
<td></td>
<td>10.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocks x AG ants</td>
<td>3</td>
<td></td>
<td>17.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>18</td>
<td></td>
<td>38.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Corrected total</strong></td>
<td>31</td>
<td></td>
<td>315.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Ant garden in southeast Perú. This nest houses the ants *Camponotus femoratus* and *Crematogaster levior* and the epiphytic plants *Peperomia macrostachya* and *Codonanthe uleana* (purple fruit). Such gardens are established when ants embed seeds of AG epiphytes into their arboreal carton nests.
Figure 2. Fate of *P. macrostachya* seeds placed at 108 sampling stations. Most *P. macrostachya* seeds remained undispersed during the bait study. Three ant species removed 390 seeds, and the AG ant *C. femoratus* was responsible for the vast majority of observed dispersal.
Figure 3. Arthropod and vertebrate exclusion experiments. (A) Close-up of a seed tray treated with both Tanglefoot and vertebrate-exclusion cage. (B) Experimental design showing the four treatments presented in random positions in 1 m² plots.
Figure 4. Removal of *P. macrostachya* seeds during exclusion experiments. Bars are proportion of seeds removed from each treatment ± SE, based on untransformed counts of seeds removed. Black bars (A) represent plots placed within AG territories, and gray bars (B) represent plots placed outside of AG territories.
Figure 5. Actual and putative seed fate pathways for *P. macrostachya* seeds. Estimated probability of events documented in the bait study, exclusion experiment, and garden census are noted by percent values next to solid lines (see below). Solid lines lacking percent values have been reported anecdotally in the literature or observed by E.Y., while pathways represented by dotted lines are proposed but undocumented. Seeds may be dispersed directly from AG plants by both ants and mammals, or they may fall to the ground. Seeds on the ground in AG territories are retrieved primarily by *C. femoratus*. If seeds undergo long distance dispersal, as when they are consumed by flying or arboreal mammals, they may also be deposited on the forest floor where they can die, be retrieved by non-AG ants or AG ants far from the original colony. Incorporation into AG carton represents a seed's best, but still unlikely, opportunity for survival.

Sources of percent values:  

**a.** 65% of *P. macrostachya* seeds were removed from within AG territories in the one-day bait study; 89% of *P. macrostachya* seeds were removed from within AG territories in the four-day exclusion experiment.  

**b.** 35% of seeds were not removed from within AG territories in the bait study; 11% were not removed in the exclusion experiment. We assume that un-removed seeds germinate in place and die.  

**c.** In the bait study, we did not observe non-AG ants removing *P. macrostachya* seeds within AG territories. However, *Dolichoderus bispinosus* may have done so, and we have occasionally seen *Pheidole astur* removing *P. macrostachya* seeds from near *C. femoratus* foraging trails.  

**d.** In the exclusion experiment, 3% of seeds were removed from trays treated with Tanglefoot, and from trays treated with both Tanglefoot and mesh cage, suggesting that mammals were of minimal importance in seed removal from AG territories.  

**e.** Here we assume that seeds deposited in vertebrate feces would occur randomly inside and outside AG territories, and would be treated the same way as seeds that had not passed through a digestive system. Hence we apply the numbers from our bait study, including bait stations inside and outside of AG territories.  

**f.** Numbers taken from our estimate of seed survival upon arrival in AG carton.
removed by AG ants
< 8%  
incorporated into carton

establish  die
> 92%f

removed by non-AG ants
11-35%b

< 1%d

removed by AG ants
65-89%a

< 1%c

destroyed

removed by non-AG ants
3%e

removed by AG ants
15%e

establish  die
< 8%  

incorporated into carton

establish  die
> 92%f

deposited in feces on branch

establish  die
< 8%  

incorporated into carton

die
die
die
die
die
discarded
discarded

die
die
die
die
die
discarded
Chapter 3

Seed odor mediates an obligate ant-plant mutualism in Amazonian rainforests

This chapter has been published in the *Proceedings of the National Academy of Sciences of the United States of America* (March 25, 2008; vol. 105, pp. 4571-4575) with coauthors Satoshi Nojima*, Christopher Häberlein‡, Stefan Schulz‡, and Coby Schal*.

*Department of Entomology and W. M. Keck Center for Behavioral Biology, North Carolina State University, Box 7613, Raleigh, NC, 27695, U.S.A. ‡Institut für Organische Chemie, Technische Universität Braunschweig, Hagenring 30, 38106 Braunschweig, Germany
Seed dispersal mutualisms are essential for the survival of diverse plant species and communities worldwide. Among invertebrates, only ants have a major role in seed dispersal, and thousands of plant species produce seeds specialized for ant dispersal in “diffuse” multi-species interactions. An outstanding but poorly understood ant-seed mutualism occurs in the Amazonian rainforest, where arboreal ants collect seeds of several epiphyte species and cultivate them in nutrient-rich nests, forming abundant and conspicuous hanging gardens known as ant-gardens (AGs). AG ants and plants are dominant members of lowland Amazonian ecosystems, and their interaction is both specific and obligate, but the means by which ants locate, recognize, and accept their mutualist seeds while rejecting other seeds, is unknown. Here we address the chemical and behavioral basis of the AG interaction. We show that workers of the AG ant Camponotus femoratus are attracted to odorants emanating from seeds of the AG plant Peperomia macrostachya, and that chemical cues also elicit seed-carrying behavior. We identify five compounds from P. macrostachya seeds that, as a blend, attract C. femoratus workers. This first report of attractive odorants from ant-dispersed seeds illustrates the intimacy and complexity of the AG mutualism, and begins to illuminate the chemical basis of this important and enigmatic interaction.
Seed dispersal mutualisms play an essential role in community regeneration and species survival (1-3). Myrmecochory, or seed dispersal by ants, occurs in some 3,000 plant species in over 80 families worldwide, and is generally a diffuse multi-species interaction mediated by seed-borne nutritional rewards called elaiosomes that are rich in proteins and lipids (4). Ants carry these seeds to their nests, consume the elaiosomes and abandon the seeds with enhanced prospects for survival and germination (4). Behavioral assays and chemical analyses indicate that ant preference for elaiosomes is mediated by characteristic nonvolatile lipids, especially 1,2-diolein, that are more typical of insect prey than of seeds (5, 6).

Myrmecochory is best described in temperate mesic forests and fire-dominated ecosystems, where it can be vital to community organization (1, 4).

Tropical ant-seed interactions, on the other hand, are poorly understood, despite the fact that ants are the most common animals in tropical moist forests (7, 8), where they play important roles in seed dispersal and viability (9-11). In the tropics, ant-dispersed seeds may lack discrete nutritional rewards, or be collected independently of them (11-13). Such seeds are best known from the Neotropical ant-gardens (AGs), an ant-plant mutualism that occurs throughout lowland Amazonia. At least two ant species are obligate gardeners that retrieve seeds of AG epiphytes—but not other seeds—embed them in arboreal carton nests, and depend upon the resulting plants for nest integrity (Fig. 1) (11-13). Ten epiphyte species in seven families are obligate ant-garden inhabitants and benefit from seed-dispersal, nutrients and defense provided by the ants (13-15). Where they occur, AG ants can be the most abundant arboreal arthropods, their territories occupying nearly 40% of forest area and their
nests providing the single most important substrate for vascular epiphytes (7, 13, 16). Despite its important role in the structure of Amazonian ecosystems, the behavioral basis of this mutualism is unknown, as are the specific cues that guide ants to retrieve certain seeds while ignoring others.

Previous observations suggested that non-nutritive chemical cues mediate ant recognition of AG seeds. AG ants do not consume the seeds themselves, but appear to use them as construction material in the nest walls (13). Although some AG seeds have elaiosomes or adhering fruit pulp that could act as nutritional rewards, ant preference for seeds does not reflect the value of these rewards, and seeds are still retrieved after rewards are removed, either by hand or by passage through a vertebrate digestive system (12, 13). This is in contrast to typical myrmecochory, which is absolutely dependent upon the elaiosome (4). In a search for chemical cues from AG seeds, essential oils of ten AG seed species were found to comprise blends of related phenolic volatiles, including methyl 2-hydroxy-6-methyl benzoate (6-MMS) in nine species (17). Nonetheless, behavioral activity of the extracted oils was not tested, and behavioral assays with synthetic compounds identified from the oils were ambiguous (18).

Here, we test the hypothesis that chemical cues mediate the interaction between AG ants and seeds, using behavioral assays with the ant Camponotus femoratus Fabricius and the plant Peperomia macrostachya (Vahl). These are the dominant AG species in southeast Perú, and they occupy more than 90% of AGs at the study site (13), EY pers. obs. In a seed-carrying behavioral assay, we applied organic solvent extracts of P. macrostachya to other
seeds that ants typically ignore (*Piper laevisatum* Kunth) and presented these test seeds, paired with solvent-treated controls, to foraging *C. femoratus*. To isolate the role of seed odor from contact chemical cues, we used a spatially controlled two-choice olfactometer assay. Finally, we used a behavior- and physiology-guided chemical analysis to pinpoint candidate compounds, and tested their behavioral activity in the olfactometer and seed-carrying assays.

**Results**

Hexane extracts of *Peperomia macrostachya* seeds elicited seed-carrying behavior in the AG ant *Camponotus femoratus*. During a total of 54 20-min trials with five ant colonies, ants retrieved 83% of *P. laevisatum* seeds that had been treated with one seed-equivalent of *P. macrostachya* extract, but retrieved only 6% of control seeds treated only with hexane (Fisher's Exact Test *P* < 0.001). In separate trials, retrieval rate increased with the dose of AG seed extract applied (Fig. 2).

In the seed carrying assay, ants could have used both olfactory and contact chemical cues to find and accept seeds. The 2-choice olfactometer assay (Fig. 3a) tested the attractiveness of seed odor alone. In this assay, control (grass) seeds were neither attractive nor repellant, but *C. femoratus* workers chose the aroma of *P. macrostachya* seeds significantly more often than that of control seeds (Fig. 3b). In a second experiment, filter paper treated with *P. macrostachya* extract was more attractive than paper treated with solvent only (Fig. 3b). During these experiments, glass wool barriers prevented ants from touching odorant sources, so ants responded to volatile compounds alone. Finally, we
removed the glass wool barriers, and ants entered the sample chambers and retrieved AG seeds. After ants began to carry seeds out of the olfactometer, subsequent ants showed enhanced preference for the AG arm of the olfactometer (Fig. 3b; mean percentage of choices for AG seeds ± s.e.m. = 59.1 ± 2.08% when only odor was available, and 71.1 ± 2.88% when contact was also allowed, t-test, P < 0.01). Thus olfactory preference for seeds can be reinforced after direct contact.

To characterize chemical cues in the complex *P. macrostachya* extract, we fractionated the behaviorally active crude extract using column chromatography and tested each fraction in the seed-carrying assay. Some behavioral activity was retained in each fraction, but only one fraction (5% ethyl acetate in hexane) was as active as the crude extract (ants retrieved both in 12 of 15 trials with 3 ant colonies, while ignoring all solvent-treated controls).

Compounds involved in olfactory attraction are likely to be physiologically perceived by *C. femoratus* antennae. To identify candidate compounds, we analyzed the active fraction using gas chromatography-electroantennographic detection (GC-EAD) (19). Eight peaks in the 5% ethyl acetate fraction elicited a consistent response from *Camponotus* antennae over 19 trials, including 3 sample concentrations processed on polar and nonpolar columns (Fig. 4). The polarity of the antenna response varied across concentrations. At the lowest concentration antennae showed sharp negative deflections in voltage, whereas at high concentrations, as in Fig. 4, some EAD responses in the latter half of the run (less volatile compounds) were positive deflections relative to the baseline voltage. Although EAG and
EAD are extensively used to reveal candidate compounds with potential behavioral effects, the electrophysiological response—presumably the summation of low frequency generator potentials from antennal neurons—remains to be elucidated (20). Eight compounds elicited consistent electrophysiological responses in ant antennae. Five of these compounds were identified by their mass spectra and coinjection of authentic standards: 3,5-dimethoxytoluene, 6-MMS, methyl o-anisate, methyl 3,5-dimethoxybenzoate, and geranyl linalool. These were detected in the active fraction at concentrations of 45 ± 6.7, 7 ± 1.4, 25 ± 3.9, 10 ± 4.0 and 221 ± 55.8 ng per seed (mean ± s.d. based on 3 analyses), respectively. Ratios and absolute amounts of these odorants varied greatly, but within an order of magnitude, among extracts.

The mass spectrum of compound 5 suggests a sesquiterpene alcohol but differs substantially from published spectra; identification awaits isolation, further analysis, and synthesis. Compounds 6 and 7 both have a molecular weight of 208 and mass spectra [base peak at m/z 81] characteristic of 2,4-dienals. They are identified as (2E,4E)-2,4-tetradecadienal and (2E,4Z)-2,4-tetradecadienal by comparison to synthetic material, which was available only after the conclusion of behavioral trials.

To determine whether the five identified compounds that elicited electrophysiological responses were also behaviorally relevant to *C. femoratus* workers, we combined them in proportions mimicking the active fraction and tested them using the olfactometer assay. *C. femoratus* workers preferred the five-component blend over solvent-treated control papers, and over papers treated with pure geranyl linalool, the most abundant
component in the blend (Fig. 5). Ant response did not differ between the two blend concentrations, nor between the blends and the crude extract (mean percentage of choices for blend ± s.e.m = 58.3 ± 2.89% for 1x blend, 61.7 ± 2.83% for 10x blend, 66.0 ± 3.89% for extract, ANOVA, $P = 0.27$).

While attractive in the olfactometer, the same five-component blend did not elicit retrieval in the seed-carrying assay. During a total of 20 20-min trials with four ant colonies, ants retrieved no *P. laevigatum* seeds that had been treated with one seed-equivalent of the blend, and only 15% of those treated with 10 seed-equivalents of the blend. In these trials, a single colony was responsible for all retrieval, including three treated seeds and one solvent blank; ants from the other three colonies investigated seeds but retrieved none. Seed removal was independent of treatment (Freeman-Halton extension of Fisher's exact test, $P > 0.05$).

**Discussion**

Our results show that chemical cues alone, rather than visual or tactile characteristics of AG seeds, are sufficient to attract AG ants and elicit the seed-collecting behavior that underlies the complex AG mutualism. We also identify a blend of five volatile components from *P. macrostachya* seeds that attract the AG ant *C. femoratus* in the olfactometer but do not elicit seed carrying behavior. To our knowledge, this is the first identification and behavioral confirmation of attractive odorants from ant-dispersed seeds.

Although AG seed chemistry has been examined previously, candidate compounds were identified based upon co-occurrence in multiple seed species, rather than occurrence in behaviorally active extracts (17). When those compounds were tested in a seed-carrying
assay, results were highly variable and ambiguous (18). The consistent positive response of *C. femoratus* to *P. macrostachya* extract in two assays is, therefore, the strongest available evidence that chemical cues mediate the AG interaction. By focusing on compounds in behaviorally active fractions, and using two behavioral assays, we quantified specific aspects of ant behavior and demonstrated attraction that would have been ambiguous or undetectable in a seed-carrying assay alone.

It is noteworthy that, despite the differences in approach, both studies identified 6-MMS as a potentially important seed recognition cue. Also outstanding for its unusual pattern of occurrence in nature, this compound has never been reported from plants other than AG seeds. It is, however, a common metabolite of fungi and insects, particularly as a semiochemical of ants (21-24). It is a trail pheromone in two myrmicine species, elicits alarm or stinging behavior in two ponerine species, and is a component of queen sex pheromone in the formicine *Polyergus rufescens*. It is widespread in the mandibular glands of male *Camponotus*, and its release coordinates mating flights in *C. herculeanus*. In *C. femoratus*, however, 6-MMS occurs only as a minor component of male mandibular glands. *C. femoratus* workers may thus be predisposed to respond to this component (17, 24).

The other four components of the attractive blend are common plant secondary metabolites, frequently reported from essential oils and floral scents. There is circumstantial evidence that, as components of floral scents, 3,5-dimethoxytoluene and methyl *o*-anisate guide foraging behavior in flower-feeding insects (25-27) while methyl *o*-anisate and methyl 3,5-dimethoxybenzoate are reported as antifeedants in pine weevil (28).
Geranyl linalool, the most abundant component of the active blend, is frequently encountered in both plants and insects. We have found this chemical in every extract of nine species of AG seeds that we have analyzed, but not in five non-AG congers (data not shown). In some bumblebees it is produced as a marking signal, is collected by orchid bees, and is a defensive secretion in *Reticulitermes* termite soldiers (29-31). In light of its defensive function, Lemaire *et al.* (32) investigated the toxicity of geranyl linalool to several species of European ants, which varied widely in their ability to withstand treatment. LD50 values ranged from 1.8 to 20,200 ng per individual, and Lemaire *et al.* hypothesized that geranyl linalool interferes with neurotransmitters of susceptible insects. The amount on a single *P. macrostachya* seed, about 220 ng, might therefore be deterrent to some species even though it is accepted by *C. femoratus*.

The active blend thus includes semiochemicals (i.e., insect pheromones and floral scents) that also have toxic and/or repellent properties for some insects. These characteristics are reasonable for a blend emitted by seeds that are dispersed by just a few ant species. In a study that will be detailed elsewhere, we compared the community of ants attracted to general food baits and AG seed baits along forest transects. We collected a total of about 70 ant species, but of these, only three species other than *C. femoratus* were observed to carry *P. macrostachya* seeds, and 85% of the observed dispersal was attributable to *C. femoratus*. Thus, in addition to attracting mutualist ants, *P. macrostachya* seed compounds might also avert inappropriate dispersers and seed predators with toxic and/or repellent properties.
The result that the five-component, electrophysiologically active blend was preferred over its major component, geranyl linalool, suggests that either behavioral activity is restricted to one or more of the phenolic minor components, or that the complete blend is necessary for attraction. Unfortunately, we were unable to conduct olfactometer assays to test all five compounds individually and in various combinations, so we cannot say how the components interact to elicit ant response. Future studies should examine the activity of each component individually and identify minimal and optimal blends.

Ant response to the blend we tested did not differ significantly from ant response to crude extract. The slightly lower activity of the blend at the natural concentration suggests a possible role for the unknowns in blend activity. Isolation, structural elucidation, and synthesis of these components is currently underway. Stereochemistry of geranyl linalool should also be further investigated in optimizing the blend. In the present study it was tested as a racemic mixture; chirality of the naturally occurring compound is unknown, and previous attempts to separate $R$ and $S$ enantiomers of geranyl linalool from natural samples have been unsuccessful (33). Nevertheless, it is noteworthy that the tested blend of just five components is as attractive, or nearly as attractive, as the crude seed extract that contains more than 150 compounds. A blend including additional or purified components would be expected to elicit an only slightly stronger ant response.

In contrast to the crude extract, however, the attractive five component blend did not elicit seed-carrying behavior in *C. femoratus*. We therefore suggest that seed-collecting is mediated by a series of different chemical cues. First, volatile compounds attract ants to an
AG seed resource. We have identified and behaviorally confirmed components that are active in this step. Second, ants handle the seeds and detect contact chemical cues that elicit carrying behavior. We have not identified these contact cues but hypothesize that they exist, because seed extracts elicit the complete behavioral sequence while synthetic attractants alone do not. We also found that some chromatographic fractions of *P. macrostachya* extract that are preferred in the seed-carrying assay are less preferred in the olfactometer, and vice versa (Chapter 4). This pattern supports the interpretation that different chemicals mediate attraction and carrying. It is perhaps not surprising that we did not pinpoint contact cues using the GC-EAD bioassay, which targets volatile compounds perceived by olfaction.

Third, when one or a few ants begin to carry the seeds, response among other ants is enhanced. This response, detected in the olfactometer assay when ants were allowed to retrieve seeds and exit the olfactometer with them, is likely mediated by ant recruitment cues, but may also involve increased diffusion of volatiles when seeds are mobilized, and/or direct interactions between seed-carrying ants and other foragers. Confirmation of this putative behavioral sequence depends upon the identification and behavioral testing of volatile unknowns and further analysis of nonvolatile seed compounds.

Nonetheless, the confirmed role of olfaction in the AG system sets it apart from other described ant-seed interactions. Elaiosomes of typical myrmecochorous seeds elicit seed-carrying with one or more non-volatile lipids, and there is evidence that olfaction does not play a role in these systems (34). The presence of an additional signaling dimension in the AG mutualism makes it similar to other insect communication systems for mate finding,
host plant location, and pollination, in which volatile cues bring an insect to the general location of its target while contact chemical cues are necessary for completion of the behavioral sequence (35-38). The complexity of the AG communication system relative to other ant-seed mutualisms may reflect its increased specificity and reciprocally obligate nature.

Materials and Methods

Study Area. All field work was conducted from October to December in 2004, 2005 and 2006, in Madre de Dios, Perú, at the Centro de Investigación y Capacitación Río Los Amigos (12º34' S, 70º6' W) in moist forest on both terra firme and seasonally inundated terrain.

Seed-Carrying Assay. Hexane extracts of *Peperomia macrostachya* seeds (see Chemical Analyses) were applied to other seeds that ants typically ignore (*Piper laevigatum*). Extract-treated seeds were paired with solvent-treated controls and presented within 5 cm of foraging trails of the AG ant *Camponotus femoratus*. Each pair of seeds was observed for 20 min and scored as carried or not carried. The synthetic blend tested included 3,5-dimethoxytoluene, 6-MMS, methyl o-anisate, methyl 3,5-dimethoxybenzoate and (E,E)-geranyl linalool at a ratio of 2:1:2:1:10 for a total of 160 ng material per seed equivalent. The blend was presented at one and 10 seed equivalents per test seed. Tests were conducted as described for extract-treated seeds except that three, not two, seeds were presented in each trial: one of each concentration and one blank.
Olfactometer Assay. The olfactometer was a Y-shaped glass tube with a 2.6 cm diameter and each of the three arms 10 cm in length. Air flow of 750 ml min\(^{-1}\) was generated using an air pump (MiDan Co., Chino, CA) that directed ambient air through a flow-meter, a coiled teflon tube, a balloon (to dampen air vibrations) and a charcoal filter before entering a Y-shaped teflon tube that split air flow evenly between the two arms of the olfactometer. Odorant sources (60 *P. macrostachya* seeds or the chemical equivalent, to mimic a typical AG seed resource, that is, a fallen *P. macrostachya* seed spike) were placed in separate Teflon sample tubes inserted in the air stream and separated from the Y-tube proper by loose glass wool plugs that prevented ants from contacting the samples. The apparatus was placed near foraging trails of *C. femoratus* such that ants entered one arm of the olfactometer and then chose between two odorant sources by turning right or left. A single trial was a pool of 30 decisions by different ants; a "decision" was counted when an ant reached the end of the basal arm and proceeded at least 5 cm down the right or left arm of the olfactometer. Trials were conducted in pairs in which the orientation of the odorant sources was reversed to control for spatial effects. The Y-tube was washed or replaced with a clean tube after each trial. The synthetic blend was the same as that described for the seed-carrying assay above. When geranyl linalool alone was tested against the blend, it was presented at the same concentration in which it occurred in the blend.

Chemical Analyses. Behaviorally active extracts were obtained by collecting mature seeds directly from *P. macrostachya* plants (the most abundant and fecund of the AG epiphytes at the study site) and soaking them in groups of 100 seeds per 3 ml GC-grade n-hexane for 30
min. Crude extract (50 seed equivalents) was concentrated under a gentle stream of air, applied to 200 mg silica gel and eluted with 3 ml each of the following solvents: hexane, 5%, 10%, 30%, and 70% ethyl acetate in hexane, ethyl acetate, and methanol.

Chromatographic procedures conducted at the field site in Perú were constrained by availability of appropriate gases (hence air instead of an inert gas) and solvents (high purity ether and dichloromethane could neither be imported nor obtained locally).

The 5% ethyl acetate fraction was further analyzed on an HP 5890 gas chromatograph equipped with flame-ionization and electroantennographic detectors (FID and EAD) interfaced with HP ChemStation software (A.09.03). Antennae of *C. femoratus* or *C. pennsylvanicus* were mounted in *Camponotus* saline (39) in capillary gold electrodes interfaced with a custom DC-amplifier (40). Analyses were conducted on two GC columns at three sample concentrations (1/2, 1/5 or 1/25 seed equivalents per analysis), for a total of 19 analyses. For the nonpolar column (DB-5, 30 m x 250 μm x 0.25 μm) the oven temperature was programmed from 80°C (2 min hold) to 300°C (20 min hold) at 10°C min⁻¹. The splitless inlet and FID were held at 320°C. For the polar column (EC-WAX, 30 m x 250 μm x 0.25 μm) the oven temperature was programmed from 80°C (1 min hold) to 260°C (20 min hold) at 10°C min⁻¹. The inlet and FID were held at 280°C. The carrier gas (He) was set at a head pressure of 135 kPa and flow rate of 2.0 ml min⁻¹.

Chemical structures of electrophysiologically active compounds were elucidated using an Agilent 6890 gas chromatograph coupled to an Agilent 5975 mass selective detector interfaced with Agilent Productivity ChemStation. Analyses were run in both EI
and CI modes, using comparable columns and programs as used for GC-EAD. The carrier gas was He at a flow of 1.2 ml min\(^{-1}\). Chemical profiles were matched to the GC-EAD/FID runs and physiologically active components identified by comparison to reference spectra in the Wiley 275 mass spectra database and by coinjection of authentic standards on both polar and nonpolar columns.

**Chemicals.** 6-MMS (methyl 2-hydroxy-6-methylbenzoate, 92%) was synthesized and purified as previously reported (17). 3,5-dimethoxytoluene (1,3-dimethoxy-5-methyl benzene, 99%), methyl 3,5-dimethoxybenzoate (99%) and methyl o-anisate (methyl 2-methoxybenzoate, 99%) were obtained from Aldrich. Geranyl linalool ((6\(E\), 10\(E\))-3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol, 63%) was purified from Acros technical grade material using silver nitrate chromatography and later obtained at 95% from Fluka.

We thank Silvia Castro, Scott Chilton, Dan Comins, Dinah Davidson, Patricia Guerra, Phil Harris, Rick Santangelo and Erick Yabar for their assistance. This work was supported by a National Science Foundation Pre-Doctoral Fellowship, United States Department of Education GAANN fellowship, Amazon Conservation Association Graduate Research Grant, a Sigma Xi Grant in Aid of Research and a North Carolina Entomological Society travel grant (to E.Y.), and by the North Carolina State University Office of International Affairs and the Blanton J. Whitmire endowment (C.S.). Permission to work in the Los Amigos
conservation concession was granted by the Instituto Nacional de Recursos Naturales (INRENA) of Perú.


**Fig. 1.** Arboreal ant-garden involving the ants *Camponotus femoratus* and *Crematogaster* cf. *limata parabiotica* and the epiphytic plants *Anthurium gracile* and *Peperomia macrostachya*. Gardens are established when ants collect seeds of their mutualist epiphytes and embed them in the nest walls, where they grow.
Fig. 2. Response of *C. femoratus* ants to different doses of *P. macrostachya* extract in seed-carrying assays. Hexane extracts of *P. macrostachya* seeds were applied to *Piper laevigatum* seeds, which ants typically ignore. Extract-treated seeds were presented within 5 cm of foraging trails of *C. femoratus* ants. *n* indicates the number of 20-min trials with six ant colonies.
Fig. 3. Behavioral tests of olfactory attractants from AG seeds. (a) Two-choice glass Y-olfactometer used to assay attraction in the field. The apparatus was placed near foraging trails of *C. femoratus*; ants entered one arm of the olfactometer and chose between two odorant sources by turning right or left in air flow generated by an air pump (top). (b) Ant responses (mean ± s.e.m.) to odorant choices presented in Y-tube olfactometer. Trials were conducted in pairs in which the orientation of the odorant sources was reversed to control for spatial effects. A single trial consisted of 30 decisions by different ants, and *n* indicates the number of trials conducted. Asterisks indicate departures from the null hypothesis of no preference, represented by the dashed line (1-sample *t*-tests, *P* ≤ 0.001); *ns* = not significant.
Figure b shows a graph depicting the percent of choices made under different conditions.

- **Offaction and contact**
  - P. macrostachya seeds
  - Grass seeds
  - P. macrostachya extract
  - Solvent control
  - n = 18

- **Offaction only, no contact**
  - P. macrostachya seeds
  - Grass seeds
  - Grass seeds
  - Nothing
  - n = 22

The graph indicates that under offaction and contact, P. macrostachya seeds and grass seeds were chosen with 100% frequency, while P. macrostachya extract and solvent control were chosen with 0% frequency. Under offaction only, no contact, grass seeds were chosen with 100% frequency, and nothing was chosen with 0% frequency.
Fig. 4. Representative GC-EAD trace of *Camponotus* antenna response to a ¼ seed equivalent of the behaviorally active fraction of *P. macrostachya* extract, separated on a nonpolar column. Highlighted GC peaks were active in 19 analyses on polar and nonpolar columns at doses as low as 0.04 seed-equivalents. The active compounds are: 1. 3,5-dimethoxytoluene; 2. 6-MMS; 3. methyl *o*-anisate; 4. methyl 3,5-dimethoxybenzoate; 5. unknown sesquiterpene alcohol; 6. (2*E*,4*Z*)-2,4-tetradecadienal; 7. (2*E*,4*E*)-2,4-tetradecadienal; 8. compounds coelute, activity could not be consistently correlated to a specific compound in analyses on different columns; 9. geranyl linalool.
Fig. 5. Behavioral confirmation that a blend of five electrophysiologically active compounds from ant-garden seeds attracts *C. femoratus* ants. Filter papers were treated with a synthetic five-component blend at 1x concentration (60 seed-equivalents, to mimic the concentration emitted by a fallen *P. macrostachya* seed spike) or 10x concentration (600 seed-equivalents) and tested in the field in a 2-choice Y-olfactometer against either solvent-treated (blank) filter papers or only geranyl linalool, the major component in the active fraction (compound 9 in Fig. 3). $P < 0.05$ for 1x concentration, and $P < 0.01$ for 10x concentration vs. blank and vs. geranyl linalool alone.
Chapter 4

Divergent chemical cues elicit seed collecting by ants in an obligate multi-species mutualism in lowland Amazonia

This chapter was prepared for submission to *Biotropica* with coauthors Patricia Guerra Bustios¹ and Coby Schal²

¹ Facultad de Ciencias Biológicas, Universidad Nacional de San Antonio Abad del Cusco, Av. de la Cultura, Nro. 733, Cusco, Perú.

² Department of Entomology and W.M. Keck Center for Behavioral Biology, North Carolina State University, Box 7613, Raleigh, NC, 27695, U.S.A.
ABSTRACT.

Throughout lowland Amazonia, arboreal ants collect seeds of several plant species and cultivate them in nutrient-rich nests, forming diverse yet species-specific symbioses known as Neotropical ant-gardens (AGs). In this obligate mutualism, ants depend on the plants for nest stability, and the plants depend on the ant nests for substrate and nutrients. AG ants and plants are abundant, dominant members of lowland Amazonian ecosystems, but the cues ants use to locate and recognize the seeds are poorly understood. To address the chemical basis of the ant-seed interaction, we surveyed seed chemistry in nine AG species and eight non-AG congeners, and detected seven phenolic and terpenoid volatiles common to seeds of all or most of nine AG species. We also analyzed seeds of three AG species (Anthurium gracile, Codonanthe uleana, and Peperomia macrostachya) using a behavior-guided fractionation procedure. At least one chromatographic fraction of each seed extract elicited retrieval behavior in the AG ant Camponotus femoratus, but the active fractions of the three plant species differed in polarity and chemical composition, indicating that the shared compounds alone were not responsible for seed-carrying behavior. Nor was a blend of the common compounds attractive to C. femoratus. Finally, we compared two different fractions of P. macrostachya extract in olfactory and seed-carrying assays. The fraction that elicited greater seed-carrying was less preferred in the olfactometer, suggesting that different chemicals mediate attraction and seed-carrying. Although AG seed species share a common interaction with ants, these results suggest that each species elicits seed-carrying with
different chemical cues, and that the seed-collecting behavior involves multiple behavioral steps mediated by different compounds.

Key words: *Anthurium gracile*; ant gardens; ant-plant mutualism; *Camponotus femoratus*; *Codonanthe uleana*, *Peperomia macrostachya*; Peru; seed dispersal

THROUGHOUT LOWLAND AMAZONIA, arboreal ants collect seeds of specific epiphytes and cultivate them in nutrient-rich carton nests, forming symbioses known as Neotropical ant-gardens (AGs). This obligate mutualism comprises a diverse but specific fauna and flora of at least five ant species in four subfamilies, and at least 15 epiphyte species in seven families (Chapter 1). The interaction between AG ants and seeds differs from other ant-seed interactions in its greater species-specificity, the subsequent long-term symbiosis of the participating species, and in its conspicuous impact on the organization of Amazonian forest communities (Wilson 1987, Davidson 1988, Nieder *et al.* 2000, Chapter 1, Chapter 2). Along a 12 km transect in southeastern Peru, AG territories occupied 16 – 39 percent of surveyed area, depending on habitat type (Davidson 1988). In the same forests, AG ants are the most frequently encountered and numerically abundant species in arboreal ant samples (Jeanne 1979, Wilson 1987, Davidson 1988, Chapter 2). The success of AG ants has been attributed to their independence from pre-existing nesting substrates, which allows them to exploit the most resource-rich arboreal microhabitats (Wilson 1987, Davidson 1988). This independence is inseparably linked to the epiphytic mutualism, because the long-term
structural integrity of the large carton nests depends on the support of epiphyte roots and nest-drying by transpiration (Yu 1994). Similarly, in Amazonian lowland forests where epiphytes are limited by substrate and nutrient availability, ant-gardens have been cited as the most important microhabitat for vascular epiphytes, due to the porous texture and enriched N, K, and P of AG carton relative to other insect carton or surrounding soil (Belin-Depoux et al. 1987, Benzing 1991, Blüthgen et al. 2001). In fact the AG epiphytes are almost never found thriving outside of ant nests (Davidson 1988, EY pers. obs.).

AGs are initiated when AG ants collect seeds of AG epiphytes, carry them to their nests and incorporate them into the carton walls (Ule 1901, Davidson 1988, Orivel & Dejean 1999). Some AG seeds bear adhering fruit pulp or elaiosomes, which ants likely eat without harming the seeds themselves (Davidson 1988). These food rewards could motivate seed collection, as lipid-rich elaiosomes do in typical temperate and sub-tropical ant-seed mutualisms (Brew et al. 1989, Beattie & Hughes 2002, Gammans et al. 2005). Several observations suggest, however, that nutritional rewards alone cannot explain ant response to AG seeds. The common AG ant _Camponotus femoratus_ (Fabricius) demonstrated consistent preferences among AG seed species, but these preferences did not reflect the assumed nutritional value of seed-borne rewards (Davidson 1988). The AG ants _C. femoratus_ and _Pachycondyla goeldii_ (Forel) also collected seeds of AG epiphytes when fruit pulp and elaiosomes had been completely removed (Orivel & Dejean 1999). On the other hand, AG seeds, even with food rewards intact, were under-utilized by, and possibly repellent to, generalist non-AG ants (Davidson 1988, Chapter 2).
Non-nutritive chemical cues are a plausible alternative mechanism for finding and recognizing AG seeds. Seidel et al. (1990) analyzed the volatile composition of AG seeds from 10 epiphyte species in seven families. Nine of these species contained the volatile compound methyl 2-hydroxy-6-methylbenzoate (6-MMS), and all released overlapping blends of four other phenolic volatiles. The role of these five compounds in seed-carrying behavior, however, was ambiguous, and *C. femoratus* rarely retrieved zeolite molecular sieves or non-AG seeds that had been treated with the five volatiles (Davidson et al. 1990, EY unpublished data). Further studies of *P. macrostachya* indicated that a relatively non-polar fraction of seed extract elicited seed-carrying behavior in *C. femoratus* (Youngsteadt et al. 2008). A blend of terpenoid and phenolic volatiles identified from that extract, including 6-MMS but not the other previously identified compounds, elicited olfactory attraction but not seed-carrying.

One of the striking features of the AG symbiosis is the taxonomic diversity of its participants, and here we ask whether seeds of different AG plants use the same or different chemistry to elicit seed-carrying behavior in the AG ant *C. femoratus*. First we identify additional compounds common to all or most AG seeds, and test these compounds in a behavioral assay. Taking a reverse approach, we also use a behavior-guided procedure to identify relevant extracts and chromatographic fractions of multiple AG seed species, and ask whether chemically similar fractions of all species are responsible for seed-carrying behavior in AG ants. Finally, to suggest whether or not olfactory attraction and seed-
carrying are mediated by the same chemical cues, we compare two fractions of *P. macrostachya* extract in both olfactory and seed-carrying assays.

**METHODS**

**STUDY AREA.**—Studies were conducted in November, 2004 and October through December of 2005 and 2006, at the Centro de Investigación y Capacitación Río Los Amigos in Madre de Dios, Peru (located at 12°34'07"S, 70°05'57"W) where AGs constructed by the ant *Camponotus femoratus* Fabricius are abundant in both floodplain and terra firme habitats. Aggregations of two to 30 nests occurred along trails at an average interval of one nest every 30 meters. *C. femoratus* occupied more than 95 percent of AGs in these habitats (*n* = 168 AGs censused); the remainder were constructed by *Azteca* species. Nine epiphyte species regularly occur in AGs at the field site, where a single nest typically hosts one to four plant species. Seeds of eight AG species were initially surveyed for chemical constituents in common; the four with the most available seeds were chosen for comparison using behavior-guided extraction and/or fractionation. These were *Peperomia macrostachya* (Vahl) (Piperaceae), which occupies 91 percent of gardens at the site; *Anthurium gracile* (Rudge) (Araceae), which occurs in 19 percent of gardens, *Codonanthe uleana* Fritsch (Gesneriaceae) in 7 percent, and *Ficus paraensis* Miq. (Moraceae) in 10 percent. All three species are considered AG-restricted, rarely occurring outside of ant nests in the Amazon: Of 674 *P. macrostachya* plants observed by Davidson (1988) at a nearby site, only five
individuals grew independently of AGs. Similarly, six of 261 *A. gracile* individuals and one of 202 *F. paraensis* individuals grew outside of AGs. *C. uleana* was not found outside AGs (Davidson 1988).

Additional AG and non-AG seeds were also collected at another site in Madre de Dios, the Estación Biológica Cocha Cashu (EBCC, 11º52'S, 71º22'W), in October, 2004.

**SURVEY OF SEED CHEMISTRY.**—In order to identify AG-specific seed chemistry, we collected seeds of nine AG seed species and eight non-AG congeners in 2004 and 2005 (Table 1), taking seeds directly from mature fruits or seed spikes. Some seeds were extracted with hexane or ethyl acetate in the field, and the extract returned to the laboratory (at NCSU) for analysis. Other seeds were stored in 1.5 ml 95 percent ethanol for transport to the lab, where the ethanol supernatant was decanted and extracted with 1.8 ml hexane. Water (0.2 ml) was added to separate the ethanol and hexane, the mixture was centrifuged at 1000 rpm for 1 min, the hexane removed, and the water-ethanol phase extracted two more times with 1.8 ml hexane. The seeds themselves were soaked for 20 min in 1 ml hexane, and all hexane extracts were combined and evaporated under a gentle stream of N\textsubscript{2} to a concentration suitable for analysis. Extracts were analyzed using gas chromatography-mass spectrometry (GC-MS). Conditions of instrumental analysis varied slightly but remained comparable over the three years of the study; a description of typical conditions follows. The GC was an Agilent 6890N, coupled to an Agilent 5975 mass selective detector, operating with Agilent Productivity ChemStation software. Manual injections of 1 μl were performed in splitless
mode. Analyses were run on a nonpolar column (DB-5MS, 30 m x 250 \( \mu \)m x 0.25 \( \mu \)m protected by 2 m of deactivated guard column), and the oven temperature went from 40°C (2 min) to 300°C (20 min) at a rate of 10°C/min. The temperature of the injector port was 300°C, and the carrier gas was He with a flow of 1.2 ml/min. Compounds common to all or most AG seed samples were provisionally identified by comparison to the Wiley 275 mass spectral database, and identifications were confirmed by coinjection with authentic standards on both nonpolar and polar columns. The polar column was an Alltech 20294 WAX, 30 m x 250 \( \mu \)m x 0.25 \( \mu \)m. In this case, the oven program went from 40°C (2 min) to 260°C (30 min) at a rate of 10°C/min. The injector port and transfer line were held at 280°C, and He was the carrier gas with a flow of 1.2 ml/min.

SEED-CARRYING ASSAY.—Extracts of AG seeds, or chromatographic fractions of those extracts (see Behavior Guided Extraction and Fractionation, below), were applied to other seeds that ants typically ignore (\emph{Piper laevigatum} Kunth). Using a 10 \( \mu \)l glass syringe, extract was applied directly to test seeds at a concentration of one AG seed equivalent per test seed, and extract-treated seeds were paired with control seeds that had been treated with an equal amount of solvent. Pairs of seeds were presented within 5 cm of foraging trails of the AG ant \emph{C. femoratus}. Each pair of seeds was observed for 20 min and scored as carried or not carried. A Fisher's exact test was used to test whether ant response was independent of seed treatment.
CHEMICALS.—6-MMS (methyl 2-hydroxy-6-methylbenzoate, 92%) was synthesized and purified as previously described (Seidel et al. 1990). Geranyl linalool [(6E,10E)-3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol, 95%] was obtained from Fluka. β-springene [(6E,10E)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene, 88%] and α-springene [(3E,6E,10E)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene, 70%] were provided by S. Schulz. Geranyl geraniol [(3E,7E,11E)-15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol, 85%] was obtained from Sigma.

OLFACTOMETER ASSAY.—The olfactometer was previously described by Youngsteadt et al. (2008). Briefly, clean air flow was split evenly to pass through two odorant sources and enter the two arms of a Y-shaped glass tube. Odorant sources consisted of 60 seed equivalents of AG seed extract or synthetic blend (a quantity that mimics natural AG seed resources such as an open fruit or fallen *P. macrostachya* seed spike) applied to clean filter paper. Controls consisted of an equal amount of solvent on filter paper. The apparatus was placed near foraging trails of *C. femoratus* such that ants entered the trunk of the olfactometer and then chose between two odorant sources by turning right or left. Ants could not touch odorant sources. A single trial was a pool of 30 decisions by different ants; a "decision" was counted when an ant reached the end of the trunk and proceeded at least 5 cm down the right or left arm of the olfactometer. Trials were conducted in pairs in which the orientation of the odorant sources was reversed to control for spatial effects. The Y-tube was replaced with a clean tube or washed with hexane, ethyl acetate, methanol and water.
after each trial. For each odorant, a one-sample t-test was used to determine whether choices for one side of the olfactometer deviated significantly from the null hypothesis of no preference, that is, 15 choices per trial.

All olfactometer trials were conducted in 2006, and included the following comparisons. To determine whether compounds common to AG seeds were attractive to *C. femoratus*, we compared a synthetic blend to solvent control. The blend consisted of the five common compounds, geranyl linalool, geranyl geraniol, β-springene, α-springene, and 6-MMS in a ratio of 10:10:1:1:0.5 based on the average amount of each compound detected in crude seed extracts that had been analyzed by that time. The geranyl geraniol and α-springene standards also contained traces of the two unidentified terpene compounds that were detected in most of the seed samples. The total mass of these compounds was set to 225 ng per seed equivalent, based on the average amount detected in crude hexane extracts that had been analyzed at the time. We selected this value, rather than the greater mass of material present in ethanol-hexane extracts, because hexane extracts had proven active in seed-carrying assays, whereas ethanol-hexane extracts had never been tested for behavioral activity. The blend was also presented in the olfactometer at a ten-fold higher concentration, *i.e.*, 2.25 µg per seed equivalent.

To suggest whether the same compounds elicit olfactory attraction and seed carrying behavior, we used both the olfactometer and seed-carrying assays to compare two different fractions (see below) of a single *P. macrostachya* extract in 2006. Again, 60 seed-
equivalents of each sample were applied to filter papers and inserted as odorant sources in the olfactometer.

**Behavior-Guided Extraction and Fractionation.**— Extracts of four species of AG seeds, *A. gracile*, *C. uleana*, *M. ulei*, and *P. macrostachya*, were subjected to seed-carrying assays in the field in 2004, 2005 and/or 2006. To obtain each extract, a group of 100 seeds of a single species were soaked in 3 ml of either GC-grade *n*-hexane, ethyl acetate, or methanol for 30 min.

In order to test ant preference for hexane and methanol extracts of *C. uleana* and *P. macrostachya* in 2004, we presented six test seeds at a time: one treated with each extract and one with each solvent. In this case we noted not only which seeds were carried after 20 minutes, but the order in which they were carried. To avoid missing values, seeds that were not carried were assigned last place in any given trial, or if *n* seeds were not carried, each was assigned the mean of the last *n* ranks. Ant preference was tested with an ANOVA on the rank order in which the seeds were carried, using PROC GLM in the SAS System for Windows, version 9.1 (SAS 2002).

For crude extracts that elicited seed-carrying behavior, 50 seed equivalents were applied to a normal-phase chromatographic column packed with 200 mg silica gel, and eluted with 3 ml each of the following solvents: hexane, 5, 10, 30, and 70 percent ethyl acetate in hexane, ethyl acetate, and methanol. Crude extracts in hexane were concentrated and applied directly to the column; extracts in ethyl acetate were evaporated to dryness in a
clean glass vial containing a small amount of silica gel, which was then returned to the column and eluted in the same manner. (Fractionation of methanol extracts was impractical under field conditions.) Pure solvents were always subjected to the same procedures in parallel for use as controls in seed-carrying assays and later laboratory analyses. Fifteen seed equivalents from each fraction were applied to 15 test seeds used in the seed-carrying assay with at least three different ant colonies. Remaining crude extract and fractions were retained for laboratory analysis. Two *A. gracile* extracts, two *P. macrostachya* extracts, and one *C. uleana* extract were subjected to this procedure.

**Chemical Analysis of Fractions.**—Each crude extract and fraction was analyzed using GC-MS, as described above. Further analyses were conducted for specific fractions as needed. Corresponding blanks were analyzed to confirm that detected compounds were of seed origin.

To test for the presence of sugars in the methanol fractions of *A. gracile* and *C. uleana*, samples were aliquoted into microreaction vials in volumes corresponding to 1, 1/5 or 1/100 seed equivalent, evaporated to dryness, and resuspended in 10 μl MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) and 10 μl pyridine. Reactions were warmed to 60°C for 30 min. Sugar standards (D-(−)-fructose, D-(+)-glucose), were derivatized in the same manner with 1 μg sugar per reaction. One or 0.5 μl of the reaction was injected in the GC-MS, equipped with the nonpolar column and with the oven programmed from 50°C (1
min) to 300°C (20 min) at a rate of 15°C min⁻¹. The inlet was held at 280°C in splitless mode. Other GC-MS parameters were as described above.

To test for the presence of amino acids in *A. gracile* and *C. uleana* fractions, samples were sent to the Molecular Structure Facility at the University of California, Davis, for analysis on a Li-citrate based Beckman 6300 amino acid analyzer. Samples (10 to 20 seed equivalents) were dried, resuspended in 200 μl AE-Cys dilution buffer, vortexed, spun down, and 50 μl loaded on the analyzer.

**RESULTS**

**THE AG SEED SIGNATURE.**—All AG seeds had three components in common, most shared an additional four, and none of these was present in non-AG congeners (Table 1, Fig. 1). Five of the seven compounds were identified as 6-MMS, α-springene, β-springene, geranyl linalool, and geranyl geraniol (complete chemical names above in Methods section under "Chemicals"). The other two were not elucidated but were close structural relatives, both characterized by electron impact mass spectra including a base peak at \( m/z \) 135, a putative molecular ion at \( m/z \) 272, and additional peaks at \( m/z \) 107, 93, 69 and 41. These compounds are believed to be allo-springenes derived from α- and β-springene (S. Schulz pers. comm.). Both unknown compounds also occurred in α-springene and geranyl geraniol standards. The seven compounds occurred in widely varying amounts and ratios in the several AG species,
ranging from barely detectable traces in some samples to the most abundant components of others (Table 1).

Olfactometer assays with a synthetic blend comprised of the five common compounds from AG seeds (and including traces of the two unknowns, as they occurred in the standards) failed to elicit olfactory attraction in *C. femoratus*. In 12 trials, the mean percent of choices for 1x blend was 53.9 ± 3.12% (± SEM, 1-sample *t* test *P* = 0.24). In 12 additional trials, ants chose 10x blend 53.3 ± 2.99% of the time (*P* = 0.29).

**BEHAVIORAL ASSAYS WITH CRUDE EXTRACTS.**—Different solvents were optimal for extracting behaviorally relevant compounds from different AG seed species. In the seed-carrying assay, when ants could choose among hexane and methanol extracts of *C. uleana* and *P. macrostachya* and solvent blanks, they preferred the methanol extract of *C. uleana* and the hexane extract of *P. macrostachya* (Fig. 2; Table 2). Table 3 shows the percent of extract-treated seeds carried, both in the above experiment and in additional trials conducted the following year. In 2005, extracts were presented individually and paired only with the corresponding blanks, so we did not observe choices among different extracts of the same seed species. In these experiments, differences in seed removal frequency were not statistically significant. Nevertheless, results for *C. uleana* and *P. macrostachya* follow the pattern predicted by the earlier preference experiment, with ants removing the most seeds treated with polar *C. uleana* extract and nonpolar *P. macrostachya* extract. Similarly, ants
removed more seeds treated with polar extract of *A. gracile* and *F. paraensis* than nonpolar extracts of the same species (Table 3).

**Fractionation and Analysis of Fractions.**—Each behaviorally active crude extract of *P. macrostachya*, *A. gracile*, and *C. uleana* that was subjected to chromatographic fractionation yielded at least one fraction that elicited seed-carrying behavior (Fig. 3). For hexane extracts of *P. macrostachya*, most fractions were somewhat active, but the 5 percent ethyl acetate in hexane fraction was nearly as effective as crude extract, particularly from one of the two extracts analyzed. For *C. uleana*, the 70 percent ethyl acetate and 100 percent ethyl acetate fractions were most active; for *A. gracile*, only the methanol fraction elicited seed-carrying, but this still resulted in less than 50 percent seed removal.

GC-MS analysis of the fractions confirmed that the active fractions of different species differed in chemical composition. Analysis of the 5 percent ethyl acetate fraction of *P. macrostachya* was described by Youngsteadt *et al.* (2008). The 70 percent ethyl acetate fraction of *C. uleana* contained compounds tentatively identified as vanillin, 4-hydroxyacetophenone, and 2,4-dihydroxyacetophenone. These identifications are based on similarity to reference spectra in the Wiley 275 mass spectral database and were not confirmed by coinjection. We could not detect anything in the 100 percent ethyl acetate fraction of the *C. uleana* extract, by GC-MS with or without derivatization with MSTFA (or with additional methods, see Discussion).
The 100 percent methanol fractions of *A. gracile* and *C. uleana* contained glucose and fructose (Table 4). These identifications were confirmed by coinjection with authentic standards derivatized using the same methods. No sugars were detected in the methanol fraction of *P. macrostachya* extract using the same methods. The 100 percent methanol fractions of *A. gracile* contained five to 11 amino acids (Table 4).

**Comparison of *P. macrostachya* Fractions in Two Assays.**—An additional *P. macrostachya* extract was fractionated in 2006. Ants carried 9 of 15 test seeds treated with the 5 percent ethyl acetate fraction. As expected, later analysis by GC-MS indicated that this fraction contained the five components previously identified as attractive (Youngsteadt *et al.* 2008), but, contrary to the previous year's fractionation results, this was not the most active fraction in the seed-carrying assay. Rather, the 30 percent ethyl acetate fraction was most active, with 12 of these 15 test seeds carried. This difference between the 5 percent and 30 percent ethyl acetate fractions was not statistically significant (Fisher's exact test $P = 0.43$).

We selected these two fractions for further comparison in the olfactometer, since we expected the 5 percent fraction to contain known volatile attractants, while the 30 percent fraction clearly contained compounds important for seed-carrying. In the olfactometer, ants preferred the 5 percent fraction over the 30 percent fraction ($61.6 \pm 3.6\%$ of choices for the 5 percent fraction, $n = 12$, 1-tailed $t$-test $P = 0.007$), a pattern opposite that observed in the seed-carrying assay.
DISCUSSION

Although all AG seed species share a common interaction with mutualist ants, these results suggest that each species elicits seed-carrying with different chemical cues. The seeds do share several volatile compounds, but we have not found evidence that these play an important role in ant behavior. Rather, no one solvent is universally ideal for obtaining seed extracts that elicit seed carrying behavior, and in fact, seed carrying is best obtained with different chromatographic fractions from the seeds of three species examined. Furthermore, when we compared two *P. macrostachya* fractions in the seed-carrying and olfactometer assays, the order of preference was reversed, a result that is consistent with our earlier suggestion that seed-collecting behavior involves multiple levels of detection mediated by different chemical cues, including both volatile attractants and contact cues (Youngsteadt *et al.* 2008).

COMMON COMPOUNDS.—We detected seven compounds common to all or most AG seeds, of which we identified five. Six of the compounds, including the two unknowns, are structurally related terpene or terpenoid volatiles; the seventh is phenolic. None of these was detected in seeds of non-AG congener species, though the terpene and terpenoid compounds have been identified in various plant essential oils (*e.g.*, Limberger *et al.* 2003, Raina *et al.* 2006, Javidnia *et al.* 2007, Zrira *et al.* 2008). All five identified compounds are known semiochemicals in other contexts. β-springene, α-springene, geranyl linalool and geranyl...
geraniol—together or separately—have been identified in labial gland secretions used as marking pheromones in the bumblebee *Bombus griseocollis*, in the Dufour's gland of the parasitoid wasp *Habrobracon hebetor*, as components of defensive secretions in *Reticulitermes* termites, as a recruitment pheromone in the ant *Ectatomma ruidum*, and as known or putative pheromones in vertebrates (Bestmann *et al.* 1995, Waterhouse *et al.* 2001, Howard *et al.* 2003, Quintana *et al.* 2003, Schulz *et al.* 2003, Bertsch *et al.* 2004).

6-MMS, on the other hand, is not reported from plants other than the AG species, where it was previously identified by Seidel *et al.* (1990). It is, however, commonly produced and perceived by ants, variously eliciting alarm, defense, trail following, or mating behaviors in different species (Longhurst *et al.* 1980, Blum *et al.* 1987, Kohl *et al.* 2000, Greenberg *et al.* 2007). 6-MMS also occurs in the heads of male *C. femoratus*, but its semiochemical function in this species is unknown (Seidel *et al.* 1990).

Indeed, both geranyl linalool and 6-MMS elicited electrophysiological response from *Camponotus* antennae, and were part of a blend identified from the 5 percent ethyl acetate fraction of *P. macrostachya* that was olfactorily attractive to *C. femoratus* (Youngsteadt *et al.* 2008). In that blend, however, 6-MMS and geranyl linalool were mixed with three other phenolic compounds (3,5-dimethoxytoluene, methyl-o-anisate, and 3,5-dimethoxybenzoate) that did not occur in seeds of other AG species. In the present study, the blend of 6-MMS, α-springene, β-springene, geranyl geraniol and geranyl linalool did not elicit olfactory attraction at either of two concentrations, a surprising result given their semiochemical potential and the prevalence of these compounds in AG seeds.
It is unlikely that the lack of ant response to the synthetic AG seed signature was due to concentrations being too high or too low, since the 1x and 10x blends encompassed the amounts typically found in seed extracts that elicited seed-carrying behavior (Table 1). There was no hint of differential ant response toward different doses of the synthetic compounds, and furthermore, the concentrations tested were comparable to those used by Youngsteadt et al. (2008) for the attractive 5-component blend. Finally, the amount of each compound varied widely among samples and species (Table 1), suggesting that a fine-tuning to a specific ratio or concentration of compounds is unlikely.

Alternatively, impurities in the synthetic compounds may have thwarted ant response. Geranyl linalool has a chiral center, but chirality of most naturally occurring samples is ambiguous (Svatos et al. 2002), and our synthetic geranyl linalool was certainly a racemic mixture. Further, our α-springene and β-springene standards were of moderate purity—although impurities in α-springene consisted primarily of β-springene and the two unknown terpenes, that is, compounds that also "belonged" in the blend. Nevertheless, trace amounts of other terpenes, terpenoids and a plasticizer were present, and may have had unintended effects on ant behavior.

We did not test a blend of 6-MMS, α-springene, β-springene, geranyl geraniol and geranyl linalool alone in the seed-carrying assay. Rather, they were included with additional volatile compounds in an early attempt to create the most complete seed-like blends possible. However, neither of two seed-like blends, based on 2004 analyses of crude extracts of A. gracile and C. uleana, elicited seed-carrying. Preliminary seed-carrying experiments with
geranyl linalool, geranyl geraniol, and 6-MMS alone or in combination at a range of
centations also suggested that these compounds alone were not worth pursuing as
 releasers of seed-carrying behavior (EY unpublished data), and we abandoned this approach
in favor of behavior-guided fractionation of highly active seed extracts.

EXTRACTS AND FRACTIONS.—The results of the behavior-guided extraction and fractionation
strongly supported the interpretation that compounds common among AG seeds are not
central to seed-carrying behavior in C. femoratus. Polar solvents (ethyl acetate and
methanol) produced the most preferred extracts of A. gracile, C. uleana, and F. paraensis,
while a nonpolar solvent (hexane) produced the preferred extract of P. macrostachya. This
observation was further borne out in the fractionation results, where each of the three seed
species examined yielded a different pattern of behavioral activity among fractions.
Although the shared compounds consistently eluted in the hexane and 5 percent ethyl acetate
fractions, regardless of species of origin, ants never preferred the hexane fraction, and only
in P. macrostachya was the 5 percent ethyl acetate fraction active. In contrast, as suggested
by ant preference for A. gracile, C. uleana and F. paraensis crude extracts made with polar
solvents, more polar fractions of these extracts were also preferred in the seed-carrying
assay. In both A. gracile and C. uleana, the methanol fraction was somewhat active, and in
C. uleana the 70 percent and 100 percent ethyl acetate fractions also elicited seed-carrying.

Although the fractionation results indicate that different classes of compounds are
responsible for seed-carrying behavior in different seed species, the identities of the specific
compounds responsible remain elusive. Monosaccharides (glucose and fructose) were present in the methanol fractions of *A. gracile* and *C. uleana*, and amino acids were also present in *A. gracile* at fairly low concentrations that were nevertheless comparable to what ants might obtain in a drop of extrafloral nectar, especially given that our ethyl acetate extracts would have removed only a small proportion of the sugars present on the seed surface. Sugars and amino acids are components of plant and hemipteran exudates that typically attract ants, and sugar and amino acid composition can contribute to species specificity and species sorting in ant-plant interactions (Bluthgen & Fiedler 2004, Heil *et al.* 2005). We did not, however, find evidence that seed sugars have a role in the AG ant-seed interaction. Monosaccharides detected in seed extracts (glucose and fructose) as well as disaccharides (sucrose) were offered on test seeds alone, together, combined with the same amino acids detected on *A. gracile* seeds, or combined with the olfactorily attractive 5-component blend of volatiles described by Youngsteadt *et al.* (2008). These seeds were almost never carried, and addition of glucose, fructose, or sucrose, together or separately, to dilute seed extracts did not increase ant preference for test seeds treated with those extracts (data not shown).

We made no attempt to behaviorally test the three phenolic volatiles tentatively identified from the 70 percent ethyl acetate fraction of *C. uleana* extract after analyzing this fraction. However, both vanillin and 2,4-dihydroxyacetophenone had been previously detected in six and five species of AG seeds, respectively, and had therefore been previously tested and did not elicit seed-carrying (Davidson *et al.* 1990, Seidel *et al.* 1990, EY
unpublished data, 2004). We could not detect anything in the 100 percent ethyl acetate fraction of *C. uleana* using the described methods, nor with HPLC-MS, nor with GC-MS after transmethylation using trimethylsilyl-diazomethane in ether, a method that would have detected fatty acids.

Even in the 5 percent ethyl acetate fraction of *P. macrostachya*, from which volatile attractants were identified (Youngsteadt *et al.* 2008), compounds that elicit seed-carrying were not isolated. We nevertheless conclude that, although seed-carrying cues have not been elucidated, they are different in *A. gracile, C. uleana*, and *P. macrostachya*. The elusive seed-carrying compounds from AG seeds may be non-volatile or thermally unstable chemicals that are not amenable to gas chromatographic analysis, and may have been missed in the GC-EAD analysis of Youngsteadt *et al.* (2008).

It is reasonable that the taxonomically diverse AG species should have arrived at their interaction with ants through different biochemical approaches—but this leaves the role of the common compounds frustratingly unexplained. The result also contrasts with other systems of seed dispersal by ants. Some 3,000 plant species in more than 80 families, mainly in the temperate and subtropical zones, rely upon ants for dispersal. Here, taxonomically diverse plants have converged upon similar morphological and chemical adaptations for ant recruitment, and those that have been analyzed typically contain 1,2-diolein, a compound that elicits seed-carrying behavior in mutualist ants (*e.g.*, Hughes *et al.* 1994, Beattie & Hughes 2002, and references therein).
VOLATILE VS. CONTACT CUES.—Earlier results suggested that, at least for *P. macrostachya*, the ant-seed interaction begins with seed-finding in response to volatile cues, followed by seed-carrying in response to contact cues, and perhaps recruitment in response to cues produced by ants already carrying seeds (Youngsteadt *et al.* 2008). The comparison of two *P. macrostachya* fractions in seed-carrying and olfactory assays is consistent with this interpretation. In 2006, the 30 percent fraction of *P. macrostachya* extract was preferred in seed-carrying assays. (The previous year, the 5 percent ethyl acetate fraction had been preferred. Analysis by GC-MS indicated that the 5 percent fractions from 2005 and 2006 had very similar volatile composition, suggesting that other undetected compounds may have been responsible for differences in seed-carrying response between years.) As expected given its chemical composition, the 5 percent fraction in 2006 was attractive in the olfactometer, and was preferred over the 30 percent fraction that had elicited a stronger seed-carrying response. Hence attraction and carrying were concentrated in different fractions and are likely mediated by different compounds, consistent with previous results that (1) volatile attractants alone did not elicit seed-carrying behavior, and (2) ant preference for *P. macrostachya* seeds in the olfactometer increased when they were allowed to touch the seeds, not only smell them (Youngsteadt *et al.* 2008).

Future studies should revisit the role of the common compounds in the behavior of AG ants, after elucidating chirality of geranyl linalool and obtaining high-purity standards. Finally, the identity of seed-carrying cues remains to be determined. Though behavior-guided fractionation is a promising approach to address this question, active fractions may
need to be further fractionated and analyzed by methods such as HPLC-MS to pinpoint nonvolatile or thermally unstable compounds that could have a role in the interaction.

ACKNOWLEDGEMENTS

We thank Michael Vega and Bruce Holst for identifying epiphytes; Erick Yabar and Silvia Castro for crucial logistical support and discussion; Jeniffer Alvarez for assistance in the field; Stefan Schulz and Christopher Häberlein for providing chemicals; Satoshi Nojima and Kevin Donahue for assistance with laboratory analyses; and the Instituto Nacional de Recursos Naturales (INRENA) of Perú for permission to work at the Cocha Cashu and Los Amigos field sites. This work was funded by an Amazon Conservation Association graduate research fellowship, a National Science Foundation predoctoral fellowship, a U. S. Department of Education GAANN biotechnology fellowship, a North Carolina State University Internationalization Seed Grant, and the Blanton J. Whitmire Endowment.

LITERATURE CITED


BLUM, M. S., L. MOREL, and H. M. FALES. 1987. Chemistry of the mandibular gland secretion of the ant Camponotus vagus Comparative Biochemistry and Physiology 86B.


Table 1. Occurrence of seven volatile compounds in AG and non-AG epiphytes.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Origin</th>
<th>Year</th>
<th>Solvent</th>
<th>Compound (percent abundance in complete extract)</th>
<th>total mass (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AG species from C. femoratus gardens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Araceae</td>
<td><em>Anthurium gracile</em></td>
<td>CC</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 7.6 3.5 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>hex</td>
<td>0.5 2.1 3.5 0.4 1.5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2005</td>
<td>EtOAc</td>
<td>6.2 7.5 1.4 35.9 1.8 0.9 17.1</td>
<td>840</td>
</tr>
<tr>
<td>Philodendron megalophyllum</td>
<td></td>
<td>CC</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 3.5 0.4 1.5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>hex</td>
<td>6.2 7.5 1.4 35.9 1.8 0.9 17.1</td>
<td>840</td>
</tr>
<tr>
<td>Bromeliaceae</td>
<td><em>Aechmea longifolia</em></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>1.4 5.8 7.7 7.2 0.7 0.3 16.5</td>
<td>37090</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>hex</td>
<td>6.7 0.8 1.1 0.1 0.1 0.3</td>
<td>740</td>
</tr>
<tr>
<td></td>
<td><em>Aechmea mertensii</em></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.2 4.9 2.2 2.1 0.5</td>
<td>5090</td>
</tr>
<tr>
<td></td>
<td><em>Epiphyllum phyllanthus</em></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.6 11.4 2.7 22.9 0.6 1.7 27.9</td>
<td>7760</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>hex</td>
<td>6.2 1.2 1.6 0.1 2.3</td>
<td>60</td>
</tr>
<tr>
<td>Cactaceae</td>
<td></td>
<td>CC</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td>Gesneriaceae</td>
<td><em>Codonanthe uleana</em></td>
<td>CC</td>
<td>2004</td>
<td>hex</td>
<td>6.2 7.5 1.4 35.9 1.8 0.9 17.1</td>
<td>840</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2005</td>
<td>EtOAc</td>
<td>6.2 7.5 1.4 35.9 1.8 0.9 17.1</td>
<td>840</td>
</tr>
<tr>
<td>Moraceae</td>
<td><em>Ficus paraensis</em></td>
<td>CC</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td>Piperaceae</td>
<td><em>Peperomia macrostachya</em></td>
<td>CC</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
<tr>
<td>Solanaceae</td>
<td><em>Markea ulei</em></td>
<td>CICRA</td>
<td>2004</td>
<td>EtOH, hex</td>
<td>0.4 2.1 37.0 3.4 0.9 8.4</td>
<td>41750</td>
</tr>
</tbody>
</table>
Table 1 Continued

<table>
<thead>
<tr>
<th>Non-AG species</th>
<th>Araceae</th>
<th>Bromeliaceae</th>
<th>Gesneriaceae</th>
<th>Moraceae</th>
<th>Piperaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anthurium bonplandii</td>
<td>Aechmea sp. 1</td>
<td>Codonanthe sp. d</td>
<td>Ficus maxima</td>
<td>Peperomia sp. 1</td>
</tr>
<tr>
<td></td>
<td>Anthurium clavigerum</td>
<td>Aechmea sp. 2</td>
<td></td>
<td></td>
<td>Peperomia sp. 2</td>
</tr>
<tr>
<td></td>
<td>CICRA 2004 EtOH, hex</td>
<td>CICRA 2004 EtOH, hex</td>
<td>CICRA 2004 EtOH, hex</td>
<td>CICRA 2004 hex</td>
<td>CICRA 2004 EtOH, hex</td>
</tr>
</tbody>
</table>

a Origin: CC = Estacion Biologica Cocha Cashu; CICRA = Centro de Investigación y Capacitación Rio Los Amigos
b Solvent: EtOH, hex: seeds were stored in ethanol, then ethanol and seeds were extracted with hexane; hex: seeds were extracted in hexane for 1 hr (2004) or 30 min (2005); EtOAC: seeds were extracted in ethyl acetate for 30 min
c Compounds: 1, 6-MMS; 2, β-springene; 3, α-springene; 4, geranyl linalool; 5, unknown #1; 6, unknown #2; 7, geranyl geraniol
d Codonanthe sp. was collected from Azteca sp. gardens in which it was the only epiphyte species present. Though this species was identified by Vega et al. (2006) as C. uleana, it was morphologically distinct from specimens found in C. femoratus gardens, and its seeds were not retrieved by C. femoratus when offered.
Table 2. Results of ANOVA on ranks, testing for effects of extract type (hexane or methanol extracts of *P. macrostachya, C. uleana*, or blanks) on the order in which ants retrieved test seeds

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>5</td>
<td>184.4</td>
<td>25.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Solvent x species interaction</td>
<td>1</td>
<td>60.2</td>
<td>42.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Colony</td>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
<td>1</td>
</tr>
<tr>
<td>Error</td>
<td>136</td>
<td>194.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>143</td>
<td>379.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Ant response to crude extracts of AG seeds prepared with different solvents and presented in seed carrying assays. Each result is the mean percent of seeds carried in $n$ trials. Five seeds were offered per trial, and each extract was tested with at least three different colonies. All treated seeds were paired with solvent-treated controls, which were retrieved <5 percent of the time.

<table>
<thead>
<tr>
<th>Seed species (year assayed)</th>
<th>mean percent carried ± SD ($n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td><em>Anthurium gracile</em> (2005)</td>
<td>40 ± 36 (6)</td>
</tr>
<tr>
<td><em>Codonanthe uleana</em> (2004)</td>
<td>53 ± 37 (6)</td>
</tr>
<tr>
<td><em>Codonanthe uleana</em> (2005)</td>
<td>53 ± 42 (3)</td>
</tr>
<tr>
<td><em>Ficus paraensis</em> (2005)</td>
<td>20 ± 35 (3)</td>
</tr>
<tr>
<td><em>Peperomia macrostachya</em> (2004)</td>
<td>73 ± 24 (6)</td>
</tr>
</tbody>
</table>
Table 4. Sugar and amino acid content of methanol fractions of *A. gracile* and *C. uleana* extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>A. gracile 1</em></th>
<th><em>A. gracile 2</em></th>
<th><em>C. uleana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugars (µg per seed)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>3.74</td>
<td>3.60 ± 0.73</td>
<td>2.07</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.29</td>
<td>5.83 ± 0.37</td>
<td>2.89</td>
</tr>
<tr>
<td><strong>Amino acids (ng per seed)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aspartic acid</td>
<td>7.4</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>threonine</td>
<td></td>
<td>1.7</td>
<td>18.1</td>
</tr>
<tr>
<td>serine</td>
<td>1.7</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>asparagine</td>
<td>6.8</td>
<td>6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>glutamic acid</td>
<td></td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td></td>
<td>2.5</td>
<td>12.6</td>
</tr>
<tr>
<td>alanine</td>
<td>2.5</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td></td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>isoleucine</td>
<td>13.6</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>leucine</td>
<td></td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>tyrosine</td>
<td>13.8</td>
<td>51.4</td>
<td></td>
</tr>
<tr>
<td>phenylalanine</td>
<td></td>
<td>10.1</td>
<td></td>
</tr>
</tbody>
</table>

*a* mean ± SD, 2 analyses
FIGURE 1. Structures of compounds frequently detected in AG seeds, but not in non-AG congeners (see Table 1).
FIGURE 2. AG ants (*C. femoratus*) preferred hexane extracts of *P. macrostachya* and methanol extracts of *C. uleana* in the seed-carrying assay. All extracts were preferred over solvent blanks. All six treatments were presented concurrently, and bars represent mean rank order in which seeds were carried during 24 30-min trials with three different ant colonies. Error bars are SEM. The most preferred seeds were carried first and have the lowest ranks.
FIGURE 3. Ants preferred different chromatographic fractions of *A. gracile*, *C. uleana* and *P. macrostachya* extracts in the seed carrying assay. Seeds were extracted in hexane (*P. macrostachya*) or ethyl acetate (*A. gracile* and *C. uleana*) and the crude extract was tested in the seed carrying assay (left). Fractions of the crude extract were eluted successively with hexane, 2 percent to 70 percent ethyl acetate in hexane, ethyl acetate, and methanol (right). Proportions of seeds carried are out of 30 seeds for *A. gracile* and *P. macrostachya*, and out of 15 seeds for *C. uleana*. Each extract or fraction was tested with at least three different ant colonies.