The Pennsylvania State University

The Graduate School

FUNGAL SOLUTIONS TO PARASITIZING ANT SOCIETIES

A Dissertation in

Entomology

by

Natalie Imirzian

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2021

The dissertation of Natalie Imirzian was reviewed and approved by the following:

David P. Hughes
Associate Professor of Entomology and Biology
Dissertation Advisor
Chair of Committee

Christina Grozinger
Distinguished Professor of Entomology

Etya Amsalem
Assistant Professor of Entomology

Ephraim Hanks
Associate Professor of Statistics

Corrie Moreau
Martha N. & John C. Moser Professor of Arthropod Biosystematics
Cornell University
Special Member

Gary W. Felton
Professor of Entomology
Head of the Department of Entomology

ABSTRACT

Fungi in the *Ophiocordyceps* (Ascomycota, Hypocreales) genus are remarkable for their ability to manipulate an ant host to leave the nest and die biting into vegetation, hence creating a 'zombie ant'. This dissertation explores *Ophiocordyceps* ant parasites in natural field conditions. Ant colonies have a suite of behaviors that protect the colony from disease and I explore how host manipulation helps the parasite evade colony defenses. I compare three zombie ant systems from different areas of the phylogeny, providing insight into the evolutionary innovations and adaptations of this fungal group. First, I examine the characteristics of a social insect society, focusing on the foraging dynamics of a commonly infected ant. Finding that most ants walk similarly, I suggest this uniform walking style prevents widespread infection, while the more exploratory ants may be at higher risk of picking up fungal spores. Next, I created a model to predict how zombie ants move in this system, then compared zombie and uninfected ant movement to demonstrate how zombie ant cadavers end up surrounding the main foraging trails. In the following two chapters, I investigate two other zombie ant systems featuring different host and parasite species. I look into how foraging ants interact with the zombie ant cadavers and find that cadavers are sometimes removed by conspecifics. These observations show how the ant host can fight back against the parasite. Overall, my dissertation illustrates the fascinating arms race between hosts and parasites, and in particular, the conflicting forces of a highly specialized parasite and a deeply protected altruistic society.

TABLE OF CONTENTS

LIST OF FIGURES vi

LIST OF TABLES viii

ACKNOWLEDGEMENTS ix

Chapter 1 Introduction 1

- The social insects 1
- Sociality and disease risk 2
- Parasite strategies for infecting social insects 3
- Overview of chapters 6

Chapter 2 Automated tracking and analysis of ant trajectories shows variation in forager exploration 9

- Abstract 9
- Introduction 10
- Methods 13
- Results 21
- Discussion 24
- Acknowledgements 28
- Figures 29

Chapter 3 An agent-based model shows zombie ants turn frequently before death 33

- Abstract 33
- Introduction 34
- Methods 36
- Results 43
- Discussion 44
- Acknowledgements 47
- Figures 48

Chapter 4 A new zombie ant behavior unraveled: aggregating on tree trunks 51

- Abstract 51
- Introduction 52
- Methods 56
- Results 59
- Discussion 61
- Acknowledgements 65
- Tables 66
- Figures 67

Chapter 5 Removal of zombie ant cadavers by conspecifics: social insect strategy limiting parasite diversification? ... 70

Abstract ... 70
Introduction ... 71
Methods ... 73
Results ... 74
Discussion ... 75
Acknowledgements ... 78
Figures ... 79

Chapter 6 Discussion ... 82

Summary ... 82
Miscellaneous observations ... 84
Future directions ... 85
Concluding remarks ... 86

References ... 88

Appendix A Supplementary Material for Chapter 2 ... 112

Description of the ant detection and tracking approach ... 112
Ant visiting map ... 114
Exploration index ... 115
Copy of published paper ... 120

Appendix B Supplementary Material for Chapter 3 ... 130

1. Calculation of model time step ... 130
2. Calculation of ant movement parameters ... 132
3. Time-on-trail analysis ... 134
4. Trail movement ... 135

Appendix C Supplementary Material for Chapter 4 ... 136

Appendix D Here today and there tomorrow: improving predictions of Desert Locust migration ... 139

Abstract ... 139
Introduction and basic biology ... 140
Locust migration ... 141
Swarm movement ... 143
Possible research directions ... 145
Conclusions ... 146
Tables ... 147
References ... 150

LIST OF FIGURES

Figure 1-1. Diversity in extended phenotypes of ant infecting *Ophiocordyceps* fungi..... 6

Figure 1-2. Overview of host and parasite species studied in dissertation research with geographic locations indicated..... 8

Figure 2-1: Trail image, trajectory overlay, and collective movement pattern 29

Figure 2-2: Distribution of trajectory straightness scores 30

Figure 2-3: Average exploration of trajectories for different colonies 31

Figure 2-4: Average exploration across time and for different straightness groups..... 32

Figure 3-1. The location of the trails and zombie ant targets for model setups..... 48

Figure 3-2. Comparison of simulated zombie ant movement values to ant movement values from the field..... 49

Figure 3-3. Parameter search results..... 50

Figure 4-1. Images of uninfected and infected *Cephalotes atratus* ants. 67

Figure 4-2. The number of infected cadavers on each graveyard tree. 68

Figure 4-3. Location of zombie ant cadavers on the surface of the trees. 69

Figure 5-1. Simplified phylogeny of the ant parasitic fungi in the *Ophiocordyceps* genus. Each fungal species is only known to parasitize one ant species. 79

Figure 5-2. Characterizing the manipulation niche for *Ophiocordyceps oecophyllae.* 80

Figure 5-3. Images of zombie green tree ants. 81

Figure 6-1. Example invertebrates observed near zombie ant cadavers. 85

Figure AA-1. Image of field camera set-up for filming ants on trails. 116

Figure AA-3. Speed of ants over time. 118

Figure AB-1. Demonstration of how ants measured from videos. 131

Figure AB-2. Distribution of mean length between steps for all ant trajectories. 132

Figure AB-3. (A) Distribution of the mean turning angles for all field ant trajectories. (B) Distribution of the turning rates for all field ant trajectories. 133

Figure AB-4. All ants in a model followed the trail for the time specified by *time-on-trail* and the number of ants reaching targets was measured by the fitness score (*f*)..... 134

Figure AC-1. The total number of trees found in all 6 graveyards divided by tree family.... 137

Figure AC-2. Distribution of trees in six different graveyards..... 138

LIST OF TABLES

Table 1-1. Convergent evolution in manipulation traits of ant parasitic taxa. 5

Table 4-1. Zombie ant cadaver interactions. 66

Table A-1. Summary of field studies that have observed ant foraging trails. 119

Table AB-1. Comparison of results for three different ways of specifying movement on trails. 135

Table AC-1. Results from Monte-Carlo simulations investigating whether the cadavers are located closer together than expected from complete spatial randomness. 136

Table D-1. Studies investigating locust movement relative to wind direction, in order of year. 147

Table D-2. Other factors that impact Desert Locust swarm movement. 148

ACKNOWLEDGEMENTS

I can't express enough gratitude to my advisor, David Hughes, for encouraging my curiosity and reminding me how lucky I am to spend my days having "conversations with dead people." More than that, I learned the importance of patience and observation. Between getting lost in reading and lost in the rainforest, I can't imagine a better way to experience graduate school. A special thanks to all members of my committee, Drs. Christina Grozinger, Etya Amsalem, Ephraim Hanks, and Corrie Moreau, for additionally supporting my development as a scientist.

My work builds upon that of previous students in the lab, particularly that of Raquel Loreto and João Araújo, and I thank them both for teaching me everything there is to know about zombie ants. Ryan Bringenberg and Emilia Sola Gracia were particularly patient and helpful when I was first starting out, and I thank Christoph Kurze for being great to work with in the field. I'm grateful to all others who have been in the lab (Lauren Quevillon, Andreas Modlmeier, Kelsee Baranowski, Dantong Zhu), but especially to Melissa Ishler and Colleen Mangold, who have been a source of stability throughout.

Many thanks to everyone else in MSC (Emily, Tyler, Allyson, Damie, Fhallon, Mario, Makaylee, Becky + many more) for always letting me talk something over while keeping me full of snacks. Thanks to Laura Drew for being a pleasure in the building, as well as tolerating my repeated borrowing of guest ID passes. I am grateful to all of the entomology department staff, but particularly to Marcia Kerschner for dealing with my many reimbursement forms.

I could not have completed chapter 2 without Yizhe Zhang, but I thank him most for our enjoyable discussions over shared meals. Hao Zheng and Danny Chen were additionally wonderful to collaborate with and I look back at my visit to Notre Dame fondly.

This work was global and my thanks are too; in Brazil I thank Antônio for his incredible knowledge of trees, sharp eye, and endless joy. Patricia and Vinicius for offering a home away from home—*muito obrigada*. Kim in South Carolina for diligently shipping memory card after memory card. Bethany for flying out and traipsing through the woods with me when I needed a friend most. Everyone in Australia, keeping me sane through days of fruitless searching, but especially Michelle at the Daintree Rainforest Observatory for her assistance and mutual excitement when I finally found zombie green ants. Knowing that the sun does rise on Sunrise Terrace helped me through many days, as I looked forward to coming home to the warmth of wonderful roommates (Erin/Kira/Senna) and hundreds of plants. Also in State College, I thank Lily for friendship and adventures that keep me grounded.

And a big thank you to my parents. Who have supported me every step of the way, while also showing me what a strong partnership looks like and instilling in me the importance of hard work, but also balance. Thanks for letting me crash the birthday party.

Finally, Rob. Whose support knows no bounds, and whose warmth shines bright. I wouldn't have met you without this work, which to me, makes this dissertation worth way more than a PhD.

This material is based upon work supported by National Science Foundation Grants IOS-1558062 and EEID 1414296 and NIH Grant R01 GM116927-0. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the NSF or NIH.

Chapter 1

Introduction

The social insects

Group living species exist across the span of eukaryotic taxa, including primates, birds, fish, and even single-celled organisms such as slime molds. While many species temporarily form groups, a group living organism can be defined as a set of individuals belonging to the same species that remain together and interact more than with other conspecific organisms (Wilson, 2000). Group living is thought to evolve when the benefits of living in a group outweigh the costs and these advantages gained from the group are known as inclusive fitness (Hamilton, 1964). Main advantages of forming a cooperative group include increased capacity to gather and access resources, improved defense from predators, as well as more mating opportunities. In some cases, group living developed into an advanced form of sociality referred to as eusociality.

Certain characteristics distinguish a eusocial society, including overlapping of generations, cooperative brood care, and reproductive division of labor (Michener, 1969; Wilson, 1971). The major eusocial groups are the ants, bees, wasps, termites, as well as a few select thrip, aphid, and beetle species (Choe & Crespi, 1997). Outside of the insects, the only organisms known to exhibit eusociality are two species of mole-rats and several species of shrimp in the genus *Synalpheus* (Duffy, 2007; Jarvis & Bennett, 1993). The eusocial insect species are known as the social insects.

Social insects have garnered fascination for centuries for their innovative and cooperative behaviors. Social insects are able to coordinate complex activities without central control, resulting in impressive nesting structures (Franks & Deneubourg, 1997; Theraulaz et al., 1998),

traffic management (Couzin & Franks, 2003; Dussutour et al., 2004; John et al., 2009), and even forms of agriculture (Mueller et al., 2005). Moreover, workers perform these behaviors while sacrificing their own reproductive fitness for the benefit of the colony. These qualities have allowed social insects to flourish all over the world, forming dense colonies of hundreds to thousands of individuals. Their ecological success warrants study into how social insects have overcome major colony threats such as infectious disease.

Sociality and disease risk

> "Although I believe not yet tested, the prediction is compelling that group-living animals will either be plagued more heavily with parasites and diseases than their solitary-living close relatives, or they will be plagued with greater expense of time and energy, and greater risk, in reducing the attacks of such organisms."
>
> – R.D. Alexander (1974)

The above quote highlights a fundamental idea in infectious disease: living in a group makes it easier for a directly transmitted parasite to infect new hosts (Anderson & May, 1979). Human disease exemplifies this tradeoff through the number of devastating pandemics sustained by humanity through time along with the global cost of disease prevention and treatment worldwide. Certain diseases of humans can quickly spread and harm a population, with notable examples including the bubonic plague of the 14th century, the 1918 influenza pandemic, and most recently the H1N1 influenza, Ebola, and COVID-19 outbreaks (Fineberg, 2014; Hays, 2005; Kaner & Schaack, 2016; Perlman, 2020). To mitigate global cost of disease, humans have developed technologies such as vaccination and antibiotics to lessen infection spread.

Analogously, social insects have developed a suite of behaviors that help protect the colony from disease, collectively known as 'social immunity' (Cremer et al., 2007). Similar to the human use of antibiotics, some social insects use antimicrobials in nest material and other places

to limit the growth of unwanted organisms (Brütsch et al., 2017; Fernández-Marín et al., 2006; Graystock & Hughes, 2011; Wang et al., 2015). Colony members will groom themselves and nestmates contaminated with fungal pathogens to reduce spore loads (Okuno et al., 2012; Reber et al., 2011). A multiple compartment nest design as well as the flexible structure of contact networks helps prevent the rapid spread of pathogens within the colony (Pie et al., 2004; Stroeymeyt et al., 2018). Additionally, social insects will remove corpses and other waste in the nest, limiting potential contact to harmful organisms (Diez et al., 2014; Sun & Zhou, 2013; Wilson-Rich et al., 2009).

While Alexander's (1974) prediction remains to be quantified regarding whether the social insects or their solitary counterparts experience more disease, the development of anti-parasite behaviors in the social insects suggests infection has posed a significant evolutionary threat to these animals. This investment in social immunity appears worthwhile, considering social insect disease epidemics are rarely reported in nature.

Parasite strategies for infecting social insects

Social insects are a widespread resource, meaning it is advantageous for parasites to evolve to exploit them despite colony defense behaviors. To focus on the ants specifically, there are over 13,000 species spread across nearly the entire globe (Bolton et al., 2006). Ants are often dominant members of an ecosystem. They comprised 69% of the insect biomass in a rainforest canopy sample (Erwin, 1989) and together with termites, were 30% of the total biomass in another rainforest sample (Fittkau & Klinge, 1973). The number of ant mimics and inquilines, from spiders (Cushing, 1997) to butterflies (Thomas & Settele, 2004) to other ants (Buschinger, 2009), demonstrates the benefits of infiltrating an ant fortress. Given the abundance and distribution of ants, it seems unlikely that parasites have not evolved the ability to circumvent

colony defense behaviors. This dissertation explores the idea that social immunity limits transmission within the nest and promotes alternative routes of infection for parasites of social insects.

An inspection of the main ant parasites demonstrates the prevalence of transmission outside of the nest as a strategy for infecting social insects. A database cataloguing ant parasites showed that 88.3% of ant parasites need the host to leave the nest for transmission to a new host (Quevillon & Hughes, 2018). Similarly, recent studies have highlighted the importance of bee pathogen transfer via flowers (Adler et al., 2018; Alger et al., 2019; Figueroa et al., 2019), including between different bee species (Graystock et al., 2015; Purkiss & Lach, 2019). Although some evidence suggests that bumblebees can avoid pathogen contaminated flowers (Fouks & Lattorff, 2011). Not much else is known about the interaction between parasites found in the environment and foraging social insect workers. The disparity between what we understand about disease prevention within the nest versus the actual transmission and behavior towards pathogenic material outside of the nest emphasizes the need for more research in this area.

Parasites that are particularly interesting in the context of social insects are manipulative parasites that alter a host's behavior in order to enhance transmission (Poulin, 2010). Manipulation might allow a parasite to bypass the social immunity behaviors of the colony by inducing a host to leave the nest to spend time in a more favorable transmission location. In fact, distantly related parasites have convergently evolved similar ways of manipulating ants (Table 1-1). These parasites induce summit disease, where ants climb up vegetation to increase the chances of transmission, and biting behavior, where the ant dies biting into vegetation, fixing itself to a potentially adaptive location. A closer look at these manipulative parasites, such as the *Ophiocordyceps* fungi*,* might provide insight into parasite strategies that promote infection of social insects.

Table 1-1. Convergent evolution in manipulation traits of ant parasitic taxa.

Host species	Parasite species	Parasite Group	Summit disease	Biting behavior	References
Formica sp.	*Pandora formicae; Pandora myrmecophaga*	Fungi Entomophthoromycota Entomopothorales	X	X	(Boer, 2008; Csata et al., 2013; Małagocka et al., 2017; Marikovsky, 1962)
Formica sp.; *Lasius sp.*	*Dicrocoelium dendriticum*	Platyhelminthes Trematoda Plagiorchiida	X	X	(Heussler et al., 1998; Krull & Mapes, 1953; Manga-González et al., 2001)
Camponotini ants	*Ophiocordyceps unilateralis sensu latu.*	Fungi Ascomycota Hypocreales	X[1]	X	(Araújo et al., 2018; Evans et al., 2011; Loreto et al., 2018)
Myrmicine ants	*Ophiocordyceps kniphofioides sensu latu*	Fungi Ascomycota Hypocreales	X	X[2]	(Araújo et al., 2018)

[1]*Ophiocordyceps blakebarnesii* infecting *Camponotus chromaiodes* is found inside logs
[2]*Ophiocordyceps daceti* infecting *Daceton armigerum* does not bite (Fig. 1-1F)

When fungi in the genus *Ophiocordyceps* (Ascomycota, Hypocreales) infect ants, the fungus induces the ant to leave the nest and die biting into vegetation (Andersen et al., 2009; Hughes et al., 2011). As infected ants act in a way that benefits the parasite to the host's detriment, infected ants are known as 'zombie ants' (Roy et al., 2006). Other species in the *Ophiocordyceps* genus that infect insects do not cause this manipulation behavior, suggesting this is an evolved strategy to infect ants (Araújo & Hughes, 2019; Wang & Yao, 2011). Moreover, each fungal species specializes on one ant host (Araújo et al., 2018; Evans et al., 2011), with the manipulation appearing to reflect the ecology and environment of the ant host (Loreto et al.,

2018). A variety of different manipulation phenotypes exist, with different fungi manipulating ants to bite into leaves, branches, tree trunks, or not even bite at all (Figure 1-1).

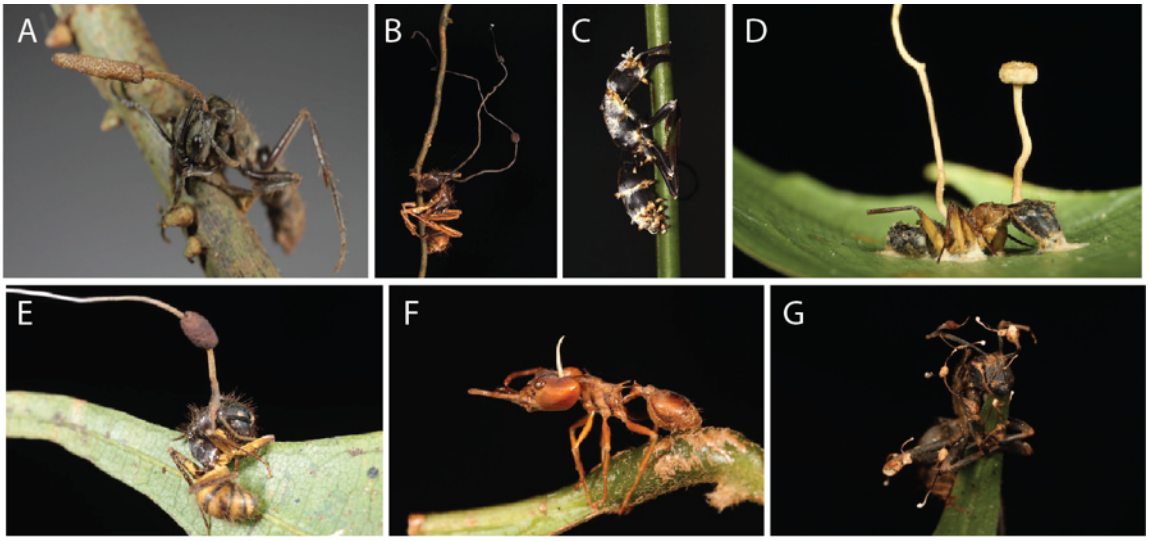


Figure 1-1. Diversity in extended phenotypes of ant infecting *Ophiocordyceps* fungi. (A) *Ophiocordyceps ponerinarum* (fungus) – *Paraponera clavata* (ant) (B) *Ophiocordyceps camponoti-renggeri* – *Camponotus renggeri* (C) undescribed *Ophiocordyceps* (with a hyperparasite) – *Paltothyreus tarsatus* (D) *Ophiocordyceps lloydii* – *Camponotus atriceps* (E) *Ophicordyceps camponoti-atriceps* – *Camponotus atriceps* (F) *Ophiocordyceps daceti* – *Daceton armigerum* (G) *Ophiocordyceps buquetii* – *Polyrachis* sp. (Photo credits: João Araújo)

Overview of chapters

This dissertation uses the zombie ant system to demonstrate how host manipulation allows a parasite to bypass social immunity behaviors and infect social insects. I use three ant-parasite pairs to investigate this: 1) the ant *Camponotus rufipes* and the fungus *Ophiocordyceps camponoti-rufipedis*, 2) the ant *Cephalotes atratus* and the fungus *Ophiocordyceps kniphofioides* and 3) the ant *Oecophylla smaragdina* and the fungus *Ophiocordyceps oecophyllae* (Fig. 1-2). Chapters 2 and 3 focus on the first paring. The parasite *Ophiocordyceps camponoti-rufipedis* is part of *Ophiocordyceps unilateralis* species complex and manipulates ants to die biting onto leaves surround the ant's foraging trails. In **chapter 2**, I look into the behavior of foraging ants on

these trails to understand the different infection risk for foraging ants. Then, I create an agent-based model using this system in **chapter 3** to understand how zombie ants are likely moving before death.

In **chapter 4**, I introduce a different zombie ant fungus, *Ophiocordyceps kniphofioides*. This fungus manipulates ants to die biting onto tree trunks, instead of the underside of leaves. I perform an ecological study where I map out the distribution of cadavers in the environment to better understand the infection cycle of the system. Moreover, I investigate the behavior of ants towards the cadavers to begin to understand how ants interact with pathogens outside of the nest. Lastly, in **chapter 5**, I investigate what is thought to be an early diverging species, *Ophiocordyceps oecophyllae*, to explain the mystery of why infected cadavers are always found damaged. The dissertation ends with a discussion in **chapter 6** on the major insights of this work, general trends in zombie ants, and future research directions.

Overall, I intend for this thesis to show how the relationship of ants to parasites satisfies both aspects of Alexander's prediction: ants invest significant energy into protecting the colony, but are still frequently plagued by parasites. An entire fungal group specializes on manipulating particular ant species, meaning ants must be beneficial to exploit. I hope more work dives deeper into how this could evolve, the fitness impact to the ant host, and the diversity in this fungal group.

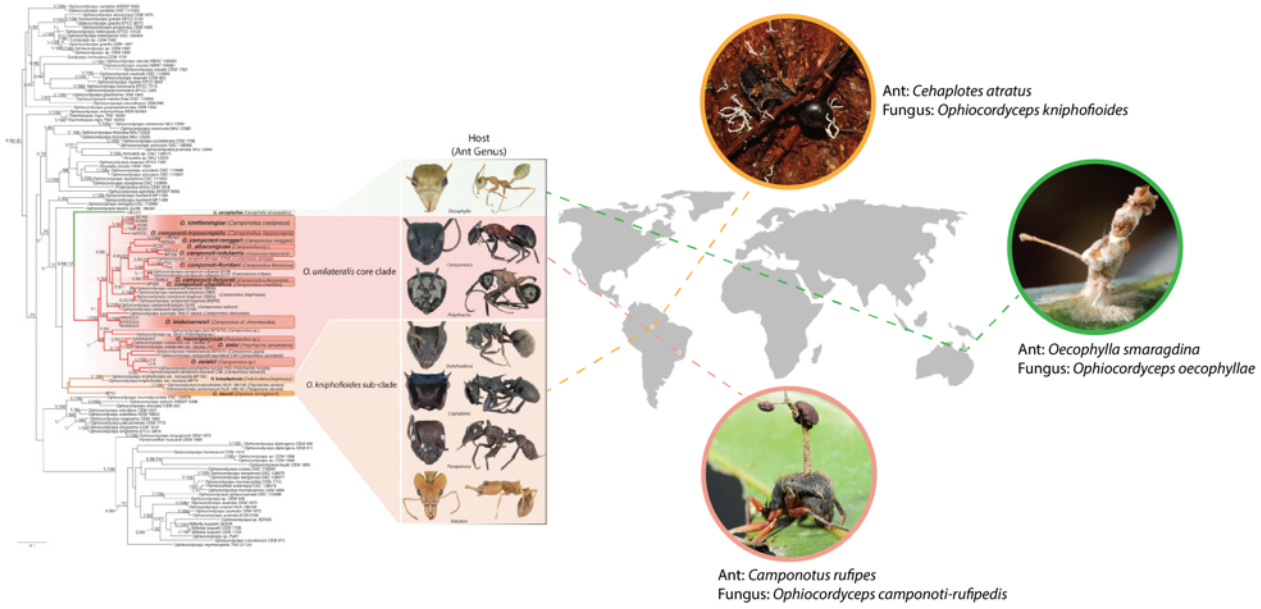


Figure 1-2. Overview of host and parasite species studied in dissertation research with geographic locations indicated.

Chapters 2 and 3 focus on *Ophiocordyceps camponoti-rufipedis* in Atlantic rainforest of Southeastern Brazil, chapter 4 investigates *O. kniphofioides* in the Brazilian Amazon, and chapter 5 looks into *O. oecophyllae* in rainforest of North Queensland, Australia. Phylogeny from Araújo et al. (2018).

Chapter 2

Automated tracking and analysis of ant trajectories shows variation in forager exploration[1]

Abstract

Determining how ant colonies optimize foraging while mitigating pathogen and predator risks provides insight into how the ants have achieved ecological success. Ants must respond to changing resource conditions, but exploration comes at a cost of higher potential exposure to threats. Fungal infected cadavers surround the main foraging trails of the carpenter ant *Camponotus rufipes,* offering a system to study how foragers behave given the persistent occurrence of disease threats. Studies on social insect foraging behavior typically require many hours of human labor due to the high density of individuals. To overcome this, we developed deep learning based computer vision algorithms to track foraging ants, frame-by-frame, from video footage shot under the natural conditions of a tropical forest floor at night. We found that most foragers walk in straight lines overlapping the same areas as other ants, but there is a subset of foragers with greater exploration. Consistency in walking behavior may protect most ants from infection, while foragers that explore unique portions of the trail may be more likely to encounter fungal spores implying a trade-off between resource discovery and risk avoidance.

[1] This chapter was published under the following citation (a copy is available in Appendix A): Imirzian, N., Zhang, Y., Kurze, C., Loreto, R.G., Chen, D.Z. and Hughes, D.P., 2019. Automated tracking and analysis of ant trajectories shows variation in forager exploration. *Scientific Reports*, *9*(1), pp.1-10.

Introduction

Resource acquisition drives animals into new territories, while threat avoidance limits where animals move. A consistent threat is the presence of infectious propagules of parasites and these are hypothesized to be major determinants of the distribution of animals in the wild (Moore, 2002). Examples of animals avoiding pathogen contaminated areas span diverse taxa, from mammals to insects, implying anti-parasite behavior is widespread (Fouks & Lattorff, 2011; Moore, 2002; Villani et al., 2002; Weinstein et al., 2018; Wynne et al., 2016). Central place foragers are interesting in the context of parasite avoidance as they must obtain food while avoiding threats with the additional constraint of returning to a defined location after each trip. For volant central place foragers, like wasps, bees, bats and birds, much of the trip is through the air likely reducing contact with infectious material. However, for taxa which walk on the ground (e.g. ants), encounters with parasite propagules are presumably higher (Boomsma et al., 2005). Unlike threats from mobile predators and competitors, parasites could directly alter movement patterns since infection occurs from a stable location on the ground. For social organisms, it would be advantageous to avoid pathogen contaminated areas in order to protect the entire colony from becoming infected.

While some ant species send workers out from the colony to forage independently, other ant species use highly coordinated groups to forage, often facilitated through chemical signaling (Hölldobler & Wilson, 1990). Group foraging via chemical trails can lead to semi-permanent trails known as 'trunk trails' (Edelstein-Keshet et al., 1995). Trunk trails stimulate research interest largely from the perspective of the self-organization behavior of ants, such as how ants regulate traffic (Couzin & Franks, 2003; Edelstein-Keshet, 1994; Fourcassié et al., 2010). Trunk trails have also been studied from the perspective of their temporal and spatial dynamics as well

as their energetic value in terms of efforts expended and resources obtained (Kost et al. 2005; Howard 2001). Yet, studies have not investigated how utilizing the same trails day after day impacts the exposure of ants to threats. Moreover, studies on ant foraging have largely occurred in a laboratory setting, and of the work that took place in the field, most studies relied on human observation or manipulated the environment in some way (see references in Table AA-1). An ant species that forages collectively and predictably in time and space would be useful to assess the relationship between trail behavior and risk avoidance.

A potential system is the carpenter ant *Camponotus rufipes* in southeastern Brazil, which forms trunk trails lasting for multiple months (Jaffe & Sanchez, 1984; Loreto et al., 2013). Colonies of this ant were recorded as having a chronic infection by the fungal parasite *Ophiocordyceps camponoti-rufipides* across 20 months (Evans, Elliot, and Hughes 2011; Loreto et al. 2014). This fungus manipulates foragers to leave the nest and die biting the underside of a leaf (Loreto et al. 2014; Evans and Samson 1984). To complete its lifecycle, the fungus must grow out of the ant cadaver and form a fruiting body that releases spores onto the ground below that will infect other ants (Evans and Samson 1984). Cadavers are found attached to leaves surrounding the ant nest (Loreto et al., 2014). The chronic nature of infection at the colony level means the spores of the pathogen are continuously in the environment from the perspective of the foragers. The spores are curved and large (80-95 microns (Evans, Elliot, and Hughes, 2011)) implying they do not travel far and land on the nearby trails once released from ant cadavers that hang above trails. Spores germinate to produce infectious secondary spores on hairs (capilliconidia) which attach to ants as they walk over them (Araújo & Hughes, 2017). Thus, infection does not require a spore to hit an ant as it walks on a trail below a cadaver. Instead, the trail substrate itself serves as the source of contamination.

Foragers of the carpenter ant *C. rufipes* mostly collect nectar from hemipteran secretions and extrafloral sources (Del-Claro & Oliveira, 1999; Jaffe & Sanchez, 1984). The exploitation of

a stable resource suggests that the most efficient way for a colony to obtain resources is for the majority of foragers to walk directly to the food source, utilizing trails near the colony entrance as a highway. However, if all foragers walked directly towards the food source, this would hinder the colony's response to changes in resource availability. We hypothesize that some individual trajectories will show evidence of searching behavior, but the majority of ants will walk directly across the trail and cover similar areas limiting the exposure of most ants to threats.

We studied the trails of seven *C. rufipes* colonies in their rainforest habitat to determine how individual ant trajectories vary in their consistency and coverage of trail space to investigate whether all foragers are at equal risk of encountering a fungal spore. Importantly, we studied ant movement on undisturbed trails, keeping pathogen risk at natural levels and including the factors undetectable to humans that influence ant foraging. We devised a system of recording trails using infrared lights and modified cameras to contend with the nocturnal foraging of this species. We then used computer vision and deep learning to automate ant tracking then characterized forager trajectories on speed, straightness, direction, and exploration.

First, we focused on the straightness of trajectories to assess the efficiency of the colony in food retrieval and to investigate whether some ants are engaged in searching behavior. Next, we analyzed the tendency of trajectories to cover unique areas of the trail through calculation of an "exploration index" of each trajectory. We predicted that most trajectories will have high straightness and low exploration scores as this increases food retrieval while limiting risk exposure. We then investigated the relationship between straightness and exploration, as well as exploration and time. We predicted that ants that walk directly across the trail are more likely to cover the same area of the trail as other ants (lower exploration), while ants with lower straightness scores are more likely to walk over a new area of the trail (high exploration). We also predicted exploration levels would be higher at the beginning of a foraging period, as this is when the pheromone trail would be the weakest. We found that some ants wander when crossing the

trail and these ants are more likely to explore a unique area of the trail, possibly increasing the flexibility of the foraging system by heightening food discovery. Conversely, covering a new area of the trail could expose wandering ants to threats other ants may avoid through following the main foraging trail.

Methods

Study site

Fieldwork took place at the Research Station of Mata do Paraíso, Universidade Federal de Viçosa, Minas Gerais, Southeast Brazil (20°48'08 S 42°52'31 W) between 10 and 25 January 2017. The carpenter ant *Camponotus rufipes* is abundant in this area, forming trails lasting multiple months (Jaffe & Sanchez, 1984; Loreto et al., 2013). The forest floor in the area of study is usually covered in 10 – 20 cm of leaf litter. Instead of traversing through the leaf litter, *C. rufipes* trails often use 'bridges' composed of woody debris, lianas, and tree branches 2 cm or more above the leaf litter (Loreto et al., 2013). Occasionally, when there are patches of clear soil (usually due to human made paths) trails would cross these areas. Ants forage at night and activity peaks in the early evening (Loreto et al., 2013).

Trail filming

Trails from seven different *C. rufipes* nests were filmed between 10 and 25 January 2017. Nests were selected based on their location and structure. Only nests found above the ground with nest material clearly visible were used. Trails were filmed before a branching point from the main trail so that ants were filmed coming directly from or towards the nest. In the case where multiple

trails came from one nest, the busiest trails were selected. The width of the branches filmed ranged from 0.8 cm to 7 cm (mean ± standard deviation; 2.97 cm ± 2.53) and the length of the area filmed for all branches was approximately 15 cm.

GoPro cameras (model: HERO 3+, GoPro, Inc., San Mateo, USA) with a modified infrared filter (RageCams.com, Michigan, USA) were used for filming. Stakes were placed 30 centimeters from the trails and 30 cm medium trigger clamps (DWHT83140, DeWalt, Towson, USA) were attached to the stakes. Cameras were attached to clamps so that cameras were approximately 30 centimeters above the trails looking down at the ants walking on the trails (Fig. AA-1). An additional camera was placed on the stake, looking sideways at the ants, to allow another perspective for behavioral analysis. Filming lasted from 19:30 to 00:00 for 4-7 nights for each trail. Timing of filming was based on previous work showing activity begins around 19:30 and peaks around 21:00 (Loreto et al., 2013). Infrared lights (IR30, CMVision, Houston, USA) were connected 12-Volt 7Ah batteries (UP1270, UniPower, São Paulo, Brazil) to allow illumination of the trail without disturbing the behavior of the ants. The camera batteries lasted for approximately 1.5 hours, so the battery was changed once in the middle of a filming period. Slight adjustments in where the trail was positioned in the video view would sometimes occur at this time. Figure 2-1a provides an example image of a trail filmed and images of the remaining trails filmed can be found in Figure AA-2.

Automated ant tracking

A total of 78 hours and 56 minutes of video were recorded for seven colonies across four nights. We developed a machine learning approach to process and analyze these videos using a deep learning based segmentation model that identified ants as they came onto the screen and tracked them as they moved across the screen (Appendix A).

Our automatic ant tracking method contains two main processes: (1) detecting ants in each image frame of all videos, and (2) building ant trajectories for every video based on the detected ants. Commonly, deep learning schemes require a large amount of labeled ground truth data for model training. Since our dataset is quite large (> 8 million image frames), we aimed to generate sufficient labeled data for training our deep learning model without incurring excessive human labeling effort. Also due to the large size of our dataset, common active learning based sample selection methods (e.g. Yang et al. (2017)) are not efficient. The goal of ant detection is to build ant movement trajectories and since ant trajectories normally span multiple consecutive frames in videos, detected ant positions in earlier frames assist with ant detection in later consecutive frames. That is, while ant detection forms a basis for building ant trajectories, trajectories of detected ants may also help ant detection. Hence, we designed our trajectory building procedure such that it not only can track detected ants but also can provide cues to indicate where (which frames and locations) there might be inconsistencies in ant trajectories and difficult scenarios for ant detection (e.g. densely clustered ants). We used such cues to select difficult cases from the frames for labeling to improve the deep learning detection model as well as the ant detection results. Therefore, our detection-tracking method consists of two rounds (with the second round improving the detection and tracking results of the first round), and each round performs two major steps, ant detection and trajectory building, as described below.

(1) Ant detection. This aims to detect ants in all the frames of the videos. We applied a novel object detection and segmentation model, Mask R-CNN (He et al., 2017), to automatically detect ants in every frame.

(2) Ant trajectory building. Given the detected ants in each frame, the next step is to form ant trajectories that connect detected ants frame-by-frame in videos. We formulated this ant

trajectory building problem as a *transportation problem*, that is, between every two consecutive frames in each video, we find an optimal transportation (for ants) that corresponds to real movement of ants. In this transportation formulation, each detected ant in frame *K* can be viewed as a 'supplier' and each detected ant in frame *K+1* can be viewed as a 'receiver'. The dissimilarity (based on spatial distance and appearance difference) between ants in two consecutive frames is a measure of how much 'cost' it would take to transport (move) one ant in frame *K* to another in frame *K+1*. The objective is to transport detected ants (as many as possible) in frame *K* to frame *K+1* with the minimum total cost. Optimal transportation based tracking methods are known to be effective for tracking sets of moving and changing objects in image sequences(Chen et al., 2016; Chen et al., 2014).

In the first round, we randomly selected frames to label as training data. This allowed us to quickly and unbiasedly obtain data samples for training a decent detection model. We then applied the trained model to all of the frames to produce ant detection results. We conducted trajectory building on detected ants to form the ant trajectories. Besides tracking ant movement, our trajectory building procedure in the first round also provided cues for identifying inconsistencies in ant trajectories and difficult cases in the frames for ant detection. In the second round, we applied training data selection to those difficult cases to find additional frames for labeling, and the enlarged training dataset thus obtained was used to re-train the Mask R-CNN detection model. The re-trained detection model was then applied to all the frames to produce the final ant detection results, which were used to build the final ant trajectories in the videos.

To identify difficult cases for additional training data selection, we used the following set of measures to capture possible errors in ant detection and trajectory results. (i) Ant speed: At a place where ants usually do not move very fast but a fast movement is suggested by the optimal transportation solution, this instance might indicate an error in ant detection. (ii) Missing ants in

the middle part of a tree branch: When the optimal transportation solution does not find a corresponding ant instance in the next frame in the interior section of a tree branch, it might suggest a missing data point in ant detection. (iii) Ant identification (ID) switching: Each detected ant was assigned an ID number; when multiple ants are seen at spatially close interaction and slight changes on the dissimilarity scores among these ants give largely different solutions for the optimal transportation problem, this might suggest an ant ID switch error. Based on these observations and measures, our trajectory building process can help identify difficult detection and tracking cases for additional training data selection to improve model performance.

Overall, we annotated 20,666 images for training the deep learning model for the ant detection task. Thus, the model is fairly robust to complex backgrounds, low contrast image areas, illumination differences. Besides relying on the training data and the robustness of Mask-RCNN model, our tracking algorithm works on the temporal information and is also robust to false-detection and miss-detection of ant. In particular, our tracking algorithm is tuned to be very robust to false-positive detections. Namely, our tracking algorithm has a strong prior/preference to discarding false-detections using temporal information. When we train and apply the Mask-RCNN model, we tolerate the Mask-RCNN model to produce some false-positive detections in order to keep the number of miss detections very low. For occasional miss-detection cases, our tracking algorithm can also recover them using temporal information.

Our automatic ant detection and tracking method extracted the x and y coordinates in pixels of detected ants in every frame and assigned each ant an identification number (Fig. 2-1a; Supplementary Video S2-1). Ant identification numbers were used to form ant trajectories used in further analysis.

Error assessment

To assess the accuracy of the computer model, we watched a subset of videos and determined the error rate. GoPro cameras automatically divide footage into 26-minute-long videos, so one night of footage at a single trail has 6 to 10 videos. This provides a way of checking the accuracy of the computer tracking at random points throughout a night. We first error checked videos from the middle of the night (when the trails should be busiest) to determine if the data from that colony was high enough quality to use in our analysis. If the average accuracy was greater than 60% for these videos, we continued to error check all videos and nights for that colony. To error check, we counted the number of ant trajectories with errors out of the first 15-30 tracked ants. The number of ant trajectories checked varied because videos from early in the foraging period sometimes had fewer ants.

To ensure consistency in the type of ant trajectories that were analyzed, trajectories beginning in the middle of the field of video view were removed. This created uniformity between all colonies and nights in the type of ants that were compared as it focused on the ants that made it from one end of the trail to the other completely in the view of the video.

Trajectory analysis

We used R version 3.4.4 and RStudio version 1.1.447 for all analyses (R Core Team, 2018; RStudio Team, 2016). Ant location data was frame-by-frame, so we used the native frame rate of the cameras (29.97 or 25 frames per second; the default setting of the cameras varied) to convert the time in frames to seconds and then used the start times of each video to convert it to real time. To convert ant location data from pixels to centimeters, we placed a ruler in each video to determine the conversion factor (Fig. AA-2).

To determine how individual ants were moving, we calculated the following variables: average speed, overall direction, time on the trail, and straightness. Average speed was taken as the total distance an ant travels while in the video over the time it takes for them to travel that distance. Overall direction was whether the ant headed away from or towards the nest which we determined based on where the ant entered and exited the video view. A variety of measures are used to determine the straightness or tortuosity of an animal's movement path (Almeida et al., 2010; Benhamou, 2004). Ant movement on trunk trails is expected to move in an oriented direction, and not be a random search path, thus we used the simplest measure, the straightness index (Almeida et al., 2010). The straightness index (ST) is a ratio between the net displacement and total path length:

$$ST = d/L;$$

where d = the distance between the beginning and end of the path and L = total path length.

To assess similarity between individual ant trajectories, we calculated an exploration index (EI) for each trajectory (Appendix A). The exploration index measures how much an individual trajectory covers unique areas of the trail space. First, we computed an Ants Visiting Map (Appendix A) for a video which estimates how frequently ants are visiting different parts of the trail. We then scored grid cells of the trail space based on how many trajectories pass through each cell. The exploration index for an individual trajectory is calculated from the scores of the grid cells that the trajectory passes through. If a trajectory mostly passes through areas of the trail space that are visited by many ants, the individual trajectory will have a low EI. To control for trajectory length, we divided the EI for a trajectory by trajectory length to get an average exploration index (AEI) for each trajectory.

Inspection of the trajectories showed that some ants performed U-turns, where they would exit the field of view from the same side that they entered on (Supplementary Video S2-2). To more accurately represent the shape of the trajectories, we broke U-turning trajectories into

two parts at the point the trajectory turned from one direction on the trail to the other and calculated straightness and exploration for the different trajectory parts individually.

Statistical analysis

A linear mixed-effects model was used to assess whether the speed of ants changes over a foraging period. The model was generated using the lmer function in the R package' lme4' (Bates et al., 2015), with speed as the response variable, time as the fixed effect, and colony and date as the random effects. The package 'lmerTest' (Kuznetsova et al., 2017) was used to generate p-values. We checked the plotted residuals to ensure homoscedasticity prior to utilizing the results of the model. We also used a linear mixed-effects model to test whether the trajectories of ants with lower straightness scores have higher exploration values. We included colony, date, and video as random effects. We fit our model with the straightness index (ST) as the fixed effect and the response variable as the average exploration index (AEI).

To analyze whether exploration differed across a foraging period, we compared the average exploration index within 30-minute intervals across the recording period. We pooled our data within 30-minute intervals to overcome discrepancies in recording times across dates. We fit a linear mixed-effects model with the interval as the fixed effect, colony and date as random effects, and the AEI for that interval as the response variable. We used a comparison of means with the Tukey method to investigate how the AEI of trajectories differed between 30-minute intervals.

Results

Automated tracking performance

The automated tracking of ants in video frames resulted in 20,230,585 data points on ant movement. The model had two types of accuracy against which it can be judged, relative to a human. The first is species accuracy (detection accuracy) which is a measure of how well the model recognized the correct species of ant. The model correctly detected *C. rufipes* ants with an accuracy of 97.86%. The model picked up other insects or species of ants on the trail (false positive) or failed to detect a *C. rufipes* ant as it went across the trail 2.14% of the time.

The second accuracy measurement is tracking accuracy. The computer had to detect *C. rufipes* ants and follow them as they moved across the screen. If an ant moved in a straight line this required the computer to recognize and track that ant for about 4 seconds or 120 frames. The computer assigned identification numbers to individual ants to follow an ant as it travelled across the screen. The machine learning model sometimes made errors in doing this. The computer may switch identification numbers when ants walked too closely together (Supplementary Video S2-3). An average of 78.70% of complete ant trajectories across all colonies had no mistakes as identified by a human observer. The tracking accuracy was the lowest for colonies MP2 (40.0%), MP11 (31.7%), and MP17 (50.6%). Identification number switches commonly happened in colonies MP2 and MP11. These trails were very thin and introduced more challenges in determining the trajectories of individual ants, so they were removed from further analysis. We have additionally removed colony MP17 as an obstruction in the trail led to ants departing from the branch and walking underneath leaves (Supplementary Video S2-4). Ants disappearing under leaf debris made it difficult to track an individual ant. We have made all videos and data available as we expect improved future machine learning models can make use of them.

The exclusion of these colonies brought the size of the dataset to 8,412,477 data points on ant movement from four colonies: MP1, MP6, MP10, and MP16. The large reduction in number of data points from the elimination of 3 colonies can be attributed to the configuration of these trails creating congested areas on the trails where single ants were tracked multiple times falsely inflating the number of ants and overall data points. The data points from the 4 included colonies represents the movement data for 64,498 ants. The average tracking accuracy of the remaining colonies was 81.39% (MP1: 72.0%; MP6: 82.1%; MP10: 77.2%; MP16: 92.1%). Most errors were due to an identification number switching to a different ant (8.28%). The high error rate for colony MP1 could be attributed to the darkness of the videos causing the model to miss part of an ant's trajectory or failing to detect an ant in the dark areas of the trail. If we consider only the errors where a number is on a wrong ant or a number is not on an ant, the accuracy improves greatly (overall: 90.94%; MP1: 91.5%; MP6: 88.8%; MP10: 86.6%; MP16: 96.3%). We are mainly concerned with the direction and shape of trajectories, and the main error that impacts an individual ant's trajectory is when ants switch to the wrong identification number, so the second calculation of accuracy rate is more reflective of this.

Collective movement pattern

Most ants walk on the same area of the available trail space (Fig. 2-1). The trail usage pattern is consistent between nights (Fig. 2-1c). The mean speed of all ants from all colonies and nights was 5.15 cm/s ± 1.63 (standard deviation). The average speed of the colonies ranged from 4.74 cm/s to 5.62 cm/s and within colony variability in speed was similar between colonies (mean (cm/s) ± standard deviation; MP1: 4.94±1.72; MP6: 5.58±1.62; MP10: 4.82±1.55; MP16: 4.72±1.43). The results of the linear mixed effects model showed that ant speed decreases by 0.45 cm/s ± 0.07 (standard error) throughout the night ($t_{(94)} = -6.60$, $p < 0.0001$; Fig. AA-3).

Individual trajectory analysis

Most ants walked in nearly straight lines (Fig. 2-2a). However, the negative skew of the distribution highlights the tendency of ants with low straightness scores to wander across the trail (Fig. 2-2a-b; Supplementary Video S2-5). The median straightness score across all colonies was 0.88 and was similar for each colony (MP1: 0.87; MP6: 0.89; MP10: 0.86; MP16: 0.87). We fit a beta mixture model using the R package betareg (Zeileis et al., 2016) to determine whether the distribution represents different groups. We used the Bayesian Information Criterion (BIC) to assess model fit and found that the distribution was best represented by four groups: straight (37.0%; n = 25,224; mean straightness = 0.94), semi-straight (26.2%; n = 17,840; mean straightness = 0.88), semi-curvy (30.0%; n = 20,437; mean straightness = 0.77), and curvy (6.8%; n = 4623; mean straightness = 0.49). The semi-curvy straightness group has a minimum straightness score of 0.64, so 93.2% of ants have straightness scores greater than 0.64.

The distributions of average exploration index (AEI) of trajectories differed in shape for each colony (Fig. 2-3). Across all colonies, a majority of ants showed low levels of exploration, but the positive skew of the AEI distributions indicates a group of ants that are more exploratory (Fig. 2-3). Colony 1 had the highest median AEI at 0.24, closely followed by colony 16 at 0.19. The median AEI for colony 6 and colony 10 were both approximately 0.06. There was a weak negative relationship between the straightness of a trajectory and its exploration value, as average exploration was estimated to decrease by 0.11± 3.14e-3 as straightness increases (linear mixed-effect model; $t_{(6810)} = -36.09$, $p < 2e\text{-}16$). The straightness groups significantly differed in average exploration (Fig. 2-4a; linear mixed-effect model; $t_{(6810)} = -11.03$, $p < 2e\text{-}16$). Post-hoc analysis using the Tukey Test showed that ants with curvy trajectories had the highest AEI followed by ants with semi-curvy trajectories, then ants with semi-straight trajectories, and ants with straight trajectories had the lowest AEI (linear mixed-effect model; Tukey Test; $p < 0.0001$).

Temporal pattern

Average exploration of trajectories decreased from the beginning of the foraging period to the middle of the foraging period, before increasing slightly again (Fig. 2-4b). The AEI was significantly greater (linear mixed-effect model; Tukey Test; $p < 0.0001$) at the beginning of the nigh t to all other time intervals. However, the AEI at 22:30 was significantly lower (linear mixed-effect model; Tukey Test; $p < 0.0001$) than at 23:30 or 00:00.

Discussion

Our study used an unobtrusive filming set-up to record behavioral data on more than 64,000 ants moving in a rainforest at night in an area of high disease pressure. Most ants walk in a straight line across the trail, matching our prediction of how ants might behave when using trunk trails (Figure 2-2). Similar to straightness, most ants show low levels of exploration, but a subset of ants cover unique areas of the trail (Figure 2-3). Average exploration of ants was higher at the beginning of the foraging period (Figure 2-4b). Exploration may enhance food discovery, but the low levels of exploration exhibited by the majority of ants may protect most foragers from the risks associated with venturing from the main trail.

The variation in exploration of trajectories indicates that the ants may have different foraging roles. Social insects have members of the colony known as scouts that assist in discovering and recruiting the colony to new food sources (Von Frisch 1967; Seeley 1983; Howard et al. 1996; Crawford and Rissing 1983). The higher exploration levels at the beginning of the night indicate that perhaps some of those ants are acting as scouts and recruiting ants to new food sources. Recruits should subsequently show lower levels of exploration than the scouts

as they follow a pheromone trail to the food source. Forager categories can extend beyond just scouts and recruits, as a forager's experience level and information source will alter its behavior (Biesmeijer & de Vries, 2001; Ravary et al., 2007). A forager recently recruited to a food source must engage in some searching behavior as they follow external stimuli to the food source. Meanwhile, a forager that has already made the trip to a food source is familiar with the route and should exhibit less searching behavior. Considering the variation in forager information may explain the distributions of exploration and straightness scores, showing all different levels of straightness and exploration.

A majority of the trajectories likely represent 'employed foragers' (Seeley, 2009), or foragers repeatedly exploiting a known resource, since the trails last for multiple months and usually visit a stable homopteran or honeydew secretion. Employed foragers should have lower exploration scores, as their trajectories will overlap other trajectories and this has implications for disease risk. Fungal infected cadavers surround the trunk trails of *Camponotus rufipes* in this habitat, likely dropping spores directly onto the trails below (Loreto et al., 2014). It is not possible to quantify the abundance and distribution of micron sized spores on trails in a forest, but the long term tracking of cadaver abundance and the proximity to the trails implies spore presence on the foraging trails (Loreto et al., 2014). Thus, for most ants, only the first ants walking across the trail after spores have dropped would likely pick up spores. In contrast, ants with higher exploration scores, the "explorers", are constantly more likely to encounter a spore that has not been picked up by a different ant. Through the same logic, an explorative ant has a higher chance discovering a new food source, demonstrating the benefits of this searching behavior.

We filmed only a small area of the foraging trails, providing a brief snapshot of an ant's behavior. To know whether higher exploration values represented ants that were more likely to wander from the trail and discover new food sources, one would need to follow individual ants for their entire foraging trip, which was beyond the scope of this study. In our study area,

exploration values were also impacted by the size of the trail, as ants will have higher overlap (= lower exploration) on narrower trails. The wider trails (colony 1 = 6 cm and colony 16 = 7 cm) had higher median exploration scores than the narrower trails (colony 6 = 3 cm and colony 10 = 1.7 cm). Observing ants beyond one portion of the trunk trails could remove differences between colonies on exploration based on trail size. Trail width still has implications in the context of disease exposure, however, as wider trails offer more substrate for possible spores and perhaps colonies that select larger trails have higher levels of infection.

Following individual ants for their entire foraging trip would also clarify whether individual ants vary in their level of exploration across a foraging trip. Experienced foragers tend to continue exploiting the same food source until it runs out (Seeley, 1983). Moreover, individual ants have been shown to be consistent in their exploratory behavior (d'Ettorre et al., 2017). The ants with low exploration values appear to be in retrieval mode and thus will likely continue exhibiting the same levels of exploration. Laboratory studies on trail bifurcations provide some evidence on the likelihood of ants to explore away from the main trail. For example, when Argentine ants (*Linepithema humile)* were placed in a maze to a food source, over 80% of the total traffic used the shorter path to the food source in the majority of experiments(Goss et al., 1989). Ants selecting a longer path, and ignoring pheromone signals, could represent patrollers or explorers. In a study on Pharaoh's ants (*Monomorium pharaonis*), 30% of the foragers failed to reorient themselves when placed into a trail network without other ants (Jackson et al., 2004). Perhaps these ants that fail to correctly follow the trail represent another group of foragers and match up with the exploratory group observed in our study.

Beyond food discovery and retrieval, other species of ants provide evidence of more roles within foragers, such as trail maintenance and defense. Ants were observed carrying leaves (Supplementary Video S2-6), although this could be for nest material and not trail cleaning. Another role could be maintaining the pheromone trail. For example, *Atta sexdens* minims help

with the pheromone trail instead of food transport (Evison et al., 2008). Ants were observed dragging their gaster on the trail likely depositing trail pheromone (Supplementary Video S2-7). U-turning ants have been shown to deposit pheromones at a higher rate (Hart & Jackson, 2006). Perhaps the main distinction between the groups is not in trail exploration, but in pheromone deposition, with the U-turners serving as the ants that are maintaining the strong chemical signal and allowing most ants to walk directly across the trail.

The different walking styles could also reflect defensive behavior. Smaller workers hitchhike on leaf fragments carried by larger workers in *Atta colombica* leaf-cutting ants, and this likely serves as a defense against parasitoid Phorid flies (Feener & Moss, 1990). Flies, that could possibly be parasitoids, were observed closely following ants on the trail and in some cases appearing to land which may indicate laying eggs on the ants which later become endoparasitoids (Supplementary Video S2-8). Although the prevalence of parasitoid flies attacking *C. rufipes* is unknown, we have observed adult ants infected by decapitating phorid flies in our study area (Supplementary Video S2-9). The presence of phorids could directly cause the exploring and U-turning behavior, as ants attempt to avoid flies landing on them. A follow up study could investigate this question of parasite avoidance by directly quantifying how ants behave when phorids are in the environment.

In this study, however, we focused on variability in individual forager trajectories. We found a group of foragers that explores more areas of the trail. Increased exploration increases a forager's chance of encountering a new food resource while simultaneously increasing their exposure to possible risks. The variability in forager behavior provides a possible mechanism for how a colony might mitigate risk through only having a small percentage of foragers exploring out from the safety of the main trail. The scale of our dataset, and ability to collect this data across multiple nights and colonies, increases the reliability and strength of our conclusions. Combining computational advances with behavioral observations provides a technique to

investigate the mechanisms of individual movement patterns that influence the distribution of animals in time and space.

Acknowledgements

We thank the Department of Forest Engineering at the Federal University of Viçosa for allowing us to perform this study at the Research Station of Mata do Pariso and Dr. Simon Elliot for hosting us in his laboratory. We are grateful to Charissa de Bekker who helped capture the behavior in Video S2-8. This work was supported in part by National Science Foundation Grants IOS-1558062 and EEID 1414296 to D.P.H, NSF CCF-1617735 to D.Z.C., and NIH Grant R01 GM116927-02 to D.P.H. and D.Z.C.

Supplementary videos for chapter 2: https://doi.org/10.26207/7yyw-tq36

Figures

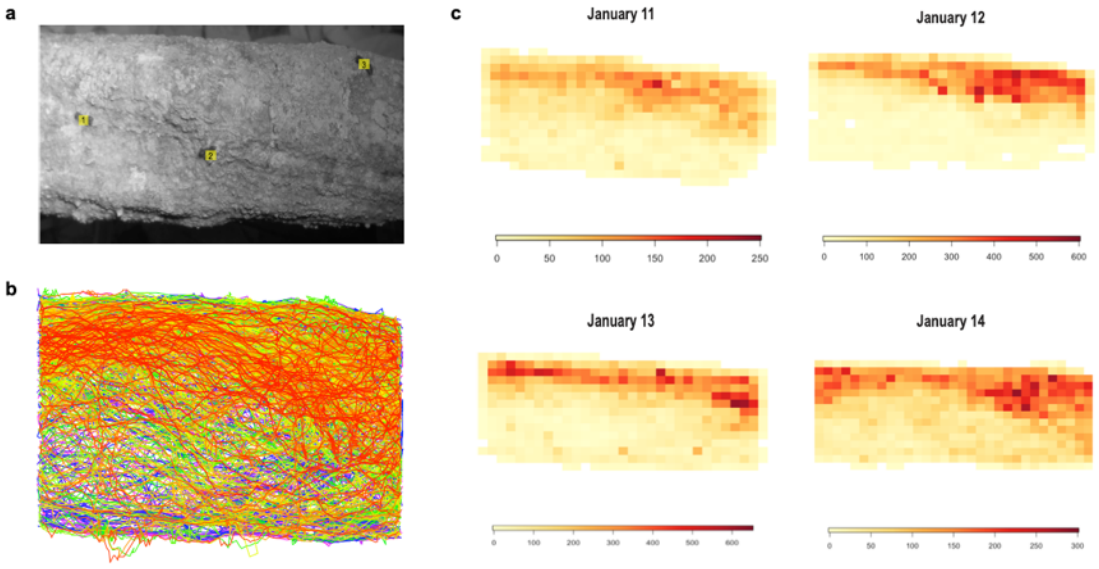


Figure 2-1: Trail image, trajectory overlay, and collective movement pattern

(a) Example trail image from GoPro footage of colony MP1. Individual ants are labeled with identification numbers. **(b)** All of the trajectories from a single night of footage (January 14) at colony MP1. Each line across the trail represents a different ant, with the different colors distinguishing between different ant tracks. **(c)** The trail space from (a) was divided into a grid with each square representing approximately 1cm2. The number of times an ant walks into a square of the grid was calculated and the darker colors represent areas of the trail that ants walked over more. Each heatmap represents a different date (January 11 through January 14) from approximately the middle of the night to control for differences in the timing of filming. Different scales were used for each night, due to variance in the number of ants that walked across the trail.

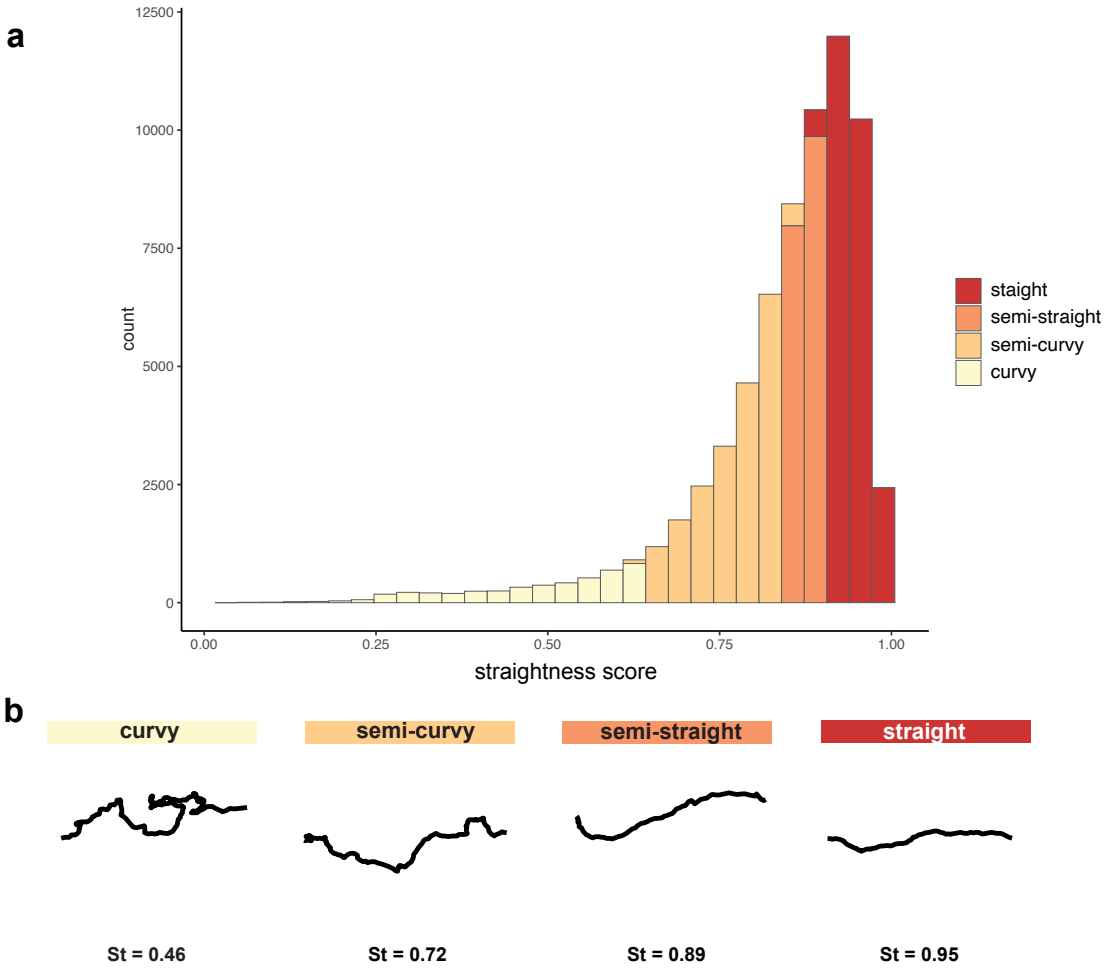


Figure 2-2: Distribution of trajectory straightness scores

(a) Histogram showing the distribution of straightness scores of ant trajectories for all nights and colonies. **(b)** Example trajectories for ants with different straightness scores. The straightness score (St) for each trajectory is included below. All 4 example trajectories were taken from the same colony and night (colony MP16 – January 15). Supplementary Video S2-5 features video of the example ants with their trajectories annotated.

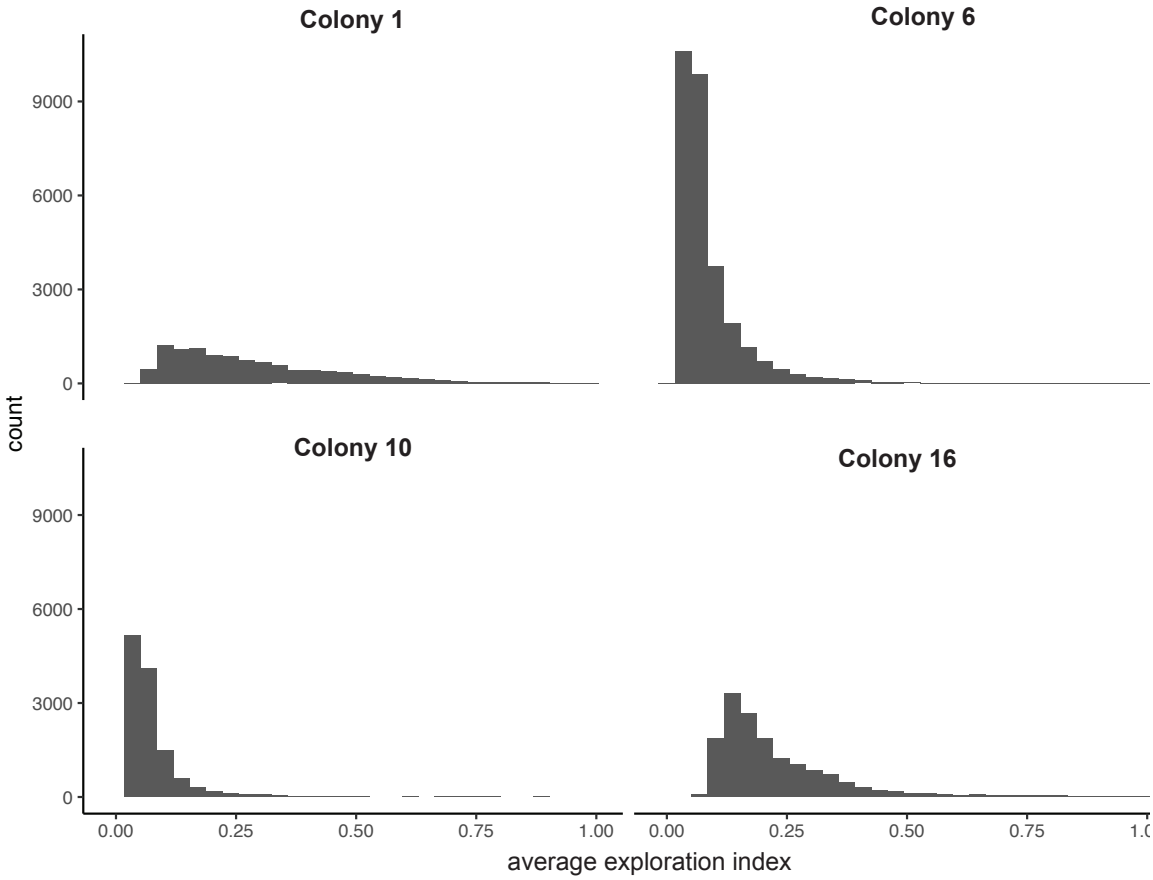


Figure 2-3: Average exploration of trajectories for different colonies

Histogram showing the distribution of the average exploration index values for all trajectories divided by colony. The average exploration index varies from 0 to 1, with 1 indicating the highest amount of exploration.

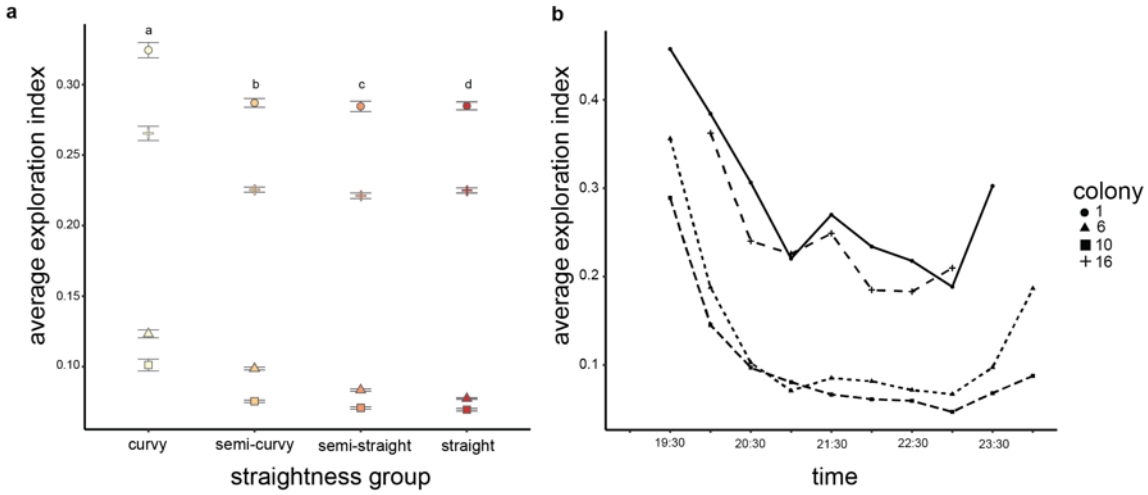


Figure 2-4: Average exploration across time and for different straightness groups

(a) The mean of the average exploration scores for the trajectories in each of the straightness groups from Fig. 2-2a. Lines indicate ± standard error of the mean. Superscripts indicate straightness groups as significantly different (linear mixed-model $p < 0.0001$). **(b)** The mean of the average exploration scores for all trajectories within each 30-minute interval across the recording period, divided by colony.

Chapter 3

An agent-based model shows zombie ants turn frequently before death[2]

Abstract

Parasites can alter the behavior of animals. Such alterations could be a byproduct of infection or actively controlled and directed by the parasite. Ants infected with zombie ant fungi (*Ophiocordyceps sp.*) show behavioral changes culminating in the ant dying while biting into vegetation. To investigate the influence of the parasite on behavioral changes, we created an agent-based model that provides a prediction of how fungal infected ants move before death. The model shows how alterations in movement, such as an increased turning rate, within the normal range of ant behavior, can lead a host from the nest to the underside of a leaf. This demonstrates the simplicity in how such behavioral changes could evolve, as the fungal parasite could benefit from the natural behavior of the host, contesting a hypothesis of highly directed manipulation.

[2] This chapter is currently under review at the *Journal of Theoretical Biology*

Introduction

Parasites impact the behavior of animals in both subtle and complex ways (Moore, 2002). In some cases, parasites act as 'puppeteers', manipulating hosts to perform behaviors adaptive to the parasite. For example, after a parasitoid emerald wasp (*Ampulex compressa*) stings its cockroach (*Periplaneta americana*) host, the wasp takes control of the cockroach's movement via the antennae, directing the cockroach to a safe location where the wasp's eggs can develop within the cockroach (Gal and Libersat, 2008). More commonly, however, behavioral changes are likely a byproduct of infection, with little adaptive benefit to the parasite (Poulin, 1995, 2010). To understand the evolution and mechanism of behavioral changes, we must distinguish where changes in host behavior fall along a spectrum from incidental changes to highly directed and adaptive manipulation.

Determining the cause of changes in host behavior requires detailed comparison between uninfected and infected host behavior. While molecular methods can elucidate changes within a host (Feldmeyer et al., 2016; Hoover et al., 2011; van Houte et al., 2013), our limited genetic toolbox for many hosts impairs our ability to connect within-host changes induced by infection to behavioral outcomes. In many systems, such direct comparisons of behavior are difficult due to an inability to distinguish infected from uninfected hosts. Hence, because a recently infected host may not show morphological changes, work on parasite behavioral changes is sometimes limited to documenting the end phenomenon, not the intermediate steps.

For instance, ants infected with zombie ant fungi in the genus *Ophiocordyceps* are identifiable as infected once the host is dead, as the host exhibits a behavior unique to infected ants where it dies biting into vegetation in the environment (Andersen et al., 2009; Hughes et al., 2011). In the hours prior to biting, infected ants show evidence of abnormal behavior, such as tremors, wandering behavior, convulsions, and low responsiveness to air, but otherwise appear

similar to uninfected ants (de Bekker et al., 2015; Hughes et al., 2011; Sakolrak, Blatrix, Sangwanit, Arnamnart, et al., 2018). Questions remain regarding the extent of parasitic influence on the pre-death behavior of the ant. The existence of clumps or 'graveyards' of infected ants in the environment (Loreto et al., 2014; Pontoppidan et al., 2009) suggests highly directed manipulation, but this might reflect the natural distribution of hosts in the environment. A study documenting how an infected host moves from the nest to the underside of a leaf is needed to understand whether zombie ants are like cockroaches on a parasitoid wasp's leash (Gal et al., 2005; Gal & Libersat, 2008) or the host ant maintains control of its movement.

While laboratory infections of zombie ants are possible (de Bekker et al., 2015; Fredericksen et al., 2017; Mangold et al., 2019), a large part of the change in behavior in this system is the spatial process of the ant moving from the nest to biting the underside of a leaf. Replicating a complex patch of rainforest where the system is naturally located in the laboratory to obtain the same pre-biting dynamics poses challenges. Moreover, as previously mentioned, infected ants show no definite signs of infection before death, limiting our ability to observe infected ants in the field. Agent-based models offer a way to overcome these challenges and could be a promising computational approach to understanding behavioral manipulation. In these simulation models, individual agents are provided rules on how to interact with each other and the environment, allowing us to test how altering these rules impacts the outcome or measured behavior.

Here, using field data on the spatial locations of zombie ant cadavers and trails (Loreto et al., 2014) and movement parameters from over 60,000 ant trajectories (Imirzian et al., 2019), we recreated the steps of an infected ant leaving the nest to biting the underside of a leaf using an agent-based model. The system, with ants originating from a defined nest and dying in an identified location, provides the information needed to quantify how a parasite impacts an

animal's movement. Comparing the simulation results with the uninfected ant movement data allows us to test the strength of behavioral changes in this host-parasite pair.

Methods

Biological info

Our model replicates the zombie ant system of the carpenter ant *Camponotus rufipes* infected with the fungal parasite *Ophiocordyceps camponoti-rufipedis*. The ant *Camponotus rufipes* forms trunk trails on tree branches, logs, and woody debris that last for multiple months (Jaffe & Sanchez, 1984; Loreto et al., 2013). Ants infected with *Ophiocordyceps camponoti-rufipedis* die biting onto the underside of leaves surrounding these trunk trails and release fungal spores onto the trails below (Loreto et al. 2014). Loreto et al. (2014) recorded the locations of *C. rufipes* zombie ant cadavers attached to leaves and the main trunk trails across seven different months for four different nests within a $200m^3$ plot (10m x 10m x 2m) with the nest at the center (Fig. 3-1). At the same field station, 64,384 *C. rufipes* ants were filmed and tracked walking on the trunk trails surrounding the ant nests (Imirzian et al., 2019). We calculated ant movement parameters from these trajectories (Appendix B.1-2) and these values formed the basis for testing our hypotheses, while the location data informed our model set-up.

The model

The model description follows the ODD (Overview, Design concepts, Details) protocol for describing agent-based models (Grimm et al., 2006, 2010). The model was created using NetLogo 6.0.3 (Wilensky, 1999).

1. Purpose

We aim to create a mechanistic model of zombie ant movement before death to demonstrate a method for investigating parasite manipulation of host behavior. This model will test whether infected ants move similarly to uninfected ants, exploring how much control the parasite has over the host.

2. Entities, state variables, and scales

The entities of the model are the ant agents and the square spatial units indicating the nest, trail, and zombie ant cadaver locations. Ants have the following state variables: *size*, *step-length*, *turning-index,* and *time-on-trail*. All ants are the same size, 0.8 cm, which was the average measured from the field (Appendix B.1). The *step-length* is the distance traveled by the ant in a model time step, while the *turning-index* controls the turning of the ant (see submodel 7.1). *Time-on-trail* determines how long an ant follows the trail after emerging from the nest.

The entire environment is represented by 100 x 100 grid cells, where each grid square corresponds to $10cm^2$, leading to a 10m x 10m world matching the field data (Fig. 3-1). Each grid square can be a location of the nest, trail, cadaver/target location, or neither. The nest is located at the center of the environment (x = 0, y = 0). All trails emerge from the nest. Trails have a width of half a patch (5 cm), which is close to the average trail width (4.4 cm) of the trails filmed in the field study (Imirzian et al., 2019). One patch marks a cadaver location, which accurately represents the range of occupiable space since biting occurs on leaves covering a larger space above the ground. The model time step is 0.2 seconds, which reflects the average time for an ant

to travel one body length (Appendix B.1). Simulation runs had a time limit of 108,000 time-steps (approx. 6 hours), the observed maximum amount of time for a zombie ant to bite after leaving the nest (de Bekker et al., 2015; Hughes et al., 2011).

3. Process overview and scheduling

The model begins with all ants at the nest (Fig. 3-1). Ants must follow trails to leave the nest. We constrict ants to exiting on trails because nests for this species are typically located above the ground with the only exit points as branches. Additionally, this ant species prefers to walk on branches over walking on the ground (Loreto et al., 2013). When walking on the trail, if the grid squares in front of an ant are not trail patches, the ant performs the smallest turn necessary to face the trail. Otherwise, the ant moves according to its *step-length* and *turning-index.* The ant walks on the trail for the length of time specified by *time-on-trail*.

At the beginning of each time step, an ant assesses whether it has reached a cadaver location. If the ant is at a cadaver location, the ant becomes a cadaver and remains at its current xy-coordinates for the rest of the model run. Ants can walk out of the bounds of the model area but cannot reenter. The model run finishes when all ants have become cadavers, exited the arena area, or the time limit has been reached (see Supp. Video S3-1 for an example run).

4. Design concepts

4.1 Basic principles

The basic principle of this model is that infected ants may move similarly to uninfected ants, or the parasite might inhibit the normal locomotion of infected ants. This model aims to test the likelihood of different zombie ant walking styles, offering a prediction on how manipulated hosts move through the environment.

4.2 Sensing

Ants can access whether their cell and their surrounding cells are trail cells or nest cells. Additionally, ants can sense when they have reached a biting location.

4.3 Stochasticity

Ants begin the model facing a random direction in a 360-degree circle at x = 0, y = 0 (the nest location). The amount an agent turns is determined by drawing a random number from a normal distribution, with 0 as the mean and the standard deviation determined from the ant's *turning-index* (see section 7.1). The amount of time an ant follows the trail is determined randomly as a number between 40 seconds and 10 minutes (see section 7.2).

4.4 Observation

The xy-coordinates of ants are recorded throughout the simulation and the final location of each ant is recorded when the time limit is reached. If an ant leaves the environment during a model run, the location where the ant exits is recorded. Additionally, we visually compared simulated trajectories with trajectories from the field with the same parameters to ensure correct implementation of the movement parameters. Validating the parameters allows us to compare the results from the simulation model with uninfected ant trajectory data from the field.

5. Initialization

Each run begins with a number of zombie ants equivalent to the number of targets in the set-up (Fig. 3-1). The input data determines the location of the trails and cadaver targets. At time = 0, all ants are located at the nest (x = 0, y = 0). All ants in a run are assigned the same *turning-index* and *step-length*. The ants are randomly assigned different *time-on-trail* values within the input range. *Time-on-trail* is at its max and will decrease every time step.

6. Input data

The model uses four different nest set-ups (Fig. 3-1). Each nest set-up is a combination of all trails recorded as coming from the nest across 10 months of field study. We used a combination of trails since zombie ants may not use the main trails used by the rest of the ants in a month. However, we wanted to limit the nest exit points to these trails since the nests are elevated above the surface of the ground and ants are likely not exiting in any direction. We used the cadaver locations from one of the first three months of field data from Loreto et al. (2014) because this represented the peak of new infections. We used month 2 for nests 4 and 5, month 3 for nest 1, and month 1 for nest 2 (Fig. 3-1). We removed cadaver points that were directly on a trail since the cadaver location would actually be above or below the trail, not directly on the trail. We removed one point for nest 2 and one point for nest 5.

7. Sub-models

7.1 Turning

We determined the turning for the ant agents through a random-normal distribution. We found that 75% of the field trajectories had a mean turning angle between -1.46 degrees (first quartile) and 1.50 degrees (third quartile) indicating most ants show no bias in direction turned (Appendix B.2). We assumed zombie ants also do not have directional bias and set the mean turning angle (μ) as equal to 0 in the distribution. We considered the standard deviation of the distribution (σ) as the *turning-index*, or standard deviation of turning angles. The *turning-index* gives an approximation for how tortuous a path is, with a low *turning-index* indicating a straight path and a high *turning-index* indicating a tortuous path. Thus, by altering the *turning-index* in the model we can investigate how turning influences the proportion of ant agents ending up on a cadaver location.

7.2 Time on trail

Considering the variation in where cadavers are found (Fig. 3-1) and the previously observed tendency of zombie ants to fall over and walk erratically (Hughes et al., 2011), different zombie ants are unlikely to spend the same amount of time following the trail once they have exited the nest. For this reason, the time an individual ant follows the trail is determined randomly within a range in the model. We decided upon the range based on the speed distribution of ants from the field trajectory analysis (Appendix B.1) and the size of the study area. Based on the minimum and maximum speeds recorded from the field, the fastest an ant could exit the arena if they kept the same pace and ran in a straight line is 30.86 seconds and the slowest an ant could exit the arena is 41.67 minutes. To narrow in on a more appropriate range, we performed preliminary model runs (Appendix B.3). From our analysis, the *time-on-trail* parameter was assigned as a random value between 40 seconds and 10 minutes, as between these values allow the ants to remain within the environment which is necessary for the ants to end up on targets.

7.3 Trail movement

Zombie ants might move similarly to uninfected ants on the trail, as they may keep up with the foraging traffic, respond to the pheromone signals on the trail, or be constrained by the boundaries of the trail. To test how ant movement on the trail impacts the outcome, we used three different setups. In setup 1, ants have the same turning rate as determined from the input parameter. Setups 2 and 3 used a two-step movement rule, where ants turned differently on the trail and off the trail. In setup 2, ants turned on the trail according to the average turning rate from the field data (low turning rate), simulating ants keeping up with foraging traffic. For setup 3, a separate input parameter determined how ants turned on the trail. We found altering the ant's turning rate on the trail relative to the turning rate off of the trail had little effect on the results (Table AB-1). Consequently, we used the simplest model for the following analysis (setup 1), where ants turn on the trail and off the trail according to the same input parameter.

Analysis

We performed a systematic search over the parameter range to find combinations that lead to a high proportion of ants ending up on targets. To distinguish between similar solutions, we measured runs based on the following: (1) number of ants on targets (2) number of ants near targets and (3) number of ants still in the arena. The proportion of ants reaching targets is the most important factor so we gave it the highest weight, followed by the number of ants near targets, and then the number of ants still in the arena. As we are not aiming to optimize ants reaching precise targets (the cadaver locations), measuring runs this way promotes solutions where ants are moving in a likely biting area at the end of the model run. We used the following equation to measure runs:

$$f = \frac{n_{target} + 0.5n_{near} + 0.25n_{arena}}{n}$$

where n is the number of ants in run, n_{target} is the number of ants that had reached a zombie ant cadaver target, n_{near} is the number of ants within 30 cm of a target, and n_{arena} is the number of ants that remained within the environment bounds. The maximum ($f = 1$) indicates that all ants reached a target location and the minimum ($f = 0$) indicates that all ants exited the arena during the model run.

We used a genetic algorithm search method to iterate through the entire parameter range to find the combinations that maximize *f*. We performed 30 searches for the 4 nest set-ups for a total of 120 searches to find the parameter distribution that most frequently leads ants near or on zombie ant cadaver locations. Within a search, each parameter combination was run 10 times in the model and the mean fitness obtained to account for stochasticity. Each parameter search evaluates the results from 500 model runs, although on some instances the search extended

beyond 500 model runs to distinguish between similar solutions. We compared the movement parameters found from the parameter search to the actual distribution of ant movement parameters to determine the probability of observing such behavior in uninfected ants.

Results

The *turning-index* obtained from all 120 parameter searches varied from 22 to 103 degrees (Fig. 3-2a). The mean *turning-index* across all searches was 73.7 degrees/step (SD = 19.4). The mean *turning-rate* that maximizes *f* did not significantly differ between nest set-ups (one-way ANOVA; $F_{(3,118)} = 0.10$, $p = 0.96$). The mean *turning-index* for nest 1 was 73.3 degrees/step (SD = 20.1), nest 2 = 72.9 degrees/step (SD = 17.3), nest 4 = 73.2 degrees/step (SD = 21.0) and nest 5 = 75.4 degrees/step (SD = 19.8). The mean *turning-index* found from the parameter searches was significantly higher than the mean *turning-index* of ants in the field data (Fig. 3-2a; Two-sample t-test; $t_{(119)} = -25.4$, $p = 5.43e\text{-}50$).

Step-length was also significantly elevated compared to the field data (Fig. 3-2b; Two-sample t-test; $t_{(119)} = -12.7$, $p = 5.52e\text{-}24$). The *step-length* obtained from the parameter searches varied from 0.5 cm to 2.9 cm with a mean of 1.6 cm. There was some variation in mean *step-length* between nests (nest 1 = 1.6 cm, nest 2 = 1.8 cm, nest 4 = 1.5 cm, and nest 5 = 1.6 cm), but the values were not significantly different (one-way ANOVA; $F_{(3, 118)} = 1.94$, $p = 0.13$).

The parameter search results also showed that a higher *turning-index* was correlated with a larger *step-length* in the combinations that maximized the fitness equation (Fig. 3-3; Pearson's correlation test; $r_{(118)} = 0.67$; $p < -2.2e\text{-}16$). The highest mean fitness (*f)* value obtained across all 120 searches was 0.71 for nest 2 and the mean score across all searches was 0.58. The nest set-ups varied in their accuracy with nest 2 performing the best (mean *f* = 0.68), followed by nest 5

(mean f = 0.55), then nest 4 (mean f = 0.55), and finally nest 1 (mean f = 0.52). High fitness scores occurred throughout the range of *step-length* and *turning-index* combinations that were obtained from the parameter searches (Fig. 3-3).

Discussion

Carpenter ants (*Camponotus rufipes*) infected with the fungus *Ophiocordyceps camponoti-rufipedis* exhibit behavioral changes following infection, with ants eventually dying while biting onto a leaf near the foraging trails. This agent-based model investigated how ant locomotion might be altered by infection prior to this biting behavior. The model suggests infection induces wandering behavior in ant hosts, as more tortuous trajectories led to a greater proportion of ant agents ended up on the biting locations. Ant agents performed a type of random walk, resulting in up to 70% of ants ending up at a zombie ant cadaver location (Fig. 3-3). Reaching a cadaver location at a high frequency was dependent on an elevated turning rate and step size compared to uninfected ants (Fig. 3-2). We suggest increased turning ensures zombie ants stay close to the nest and main trails, promoting transmission of the fungus to future ants.

Altering an infected ant's movement capabilities – whether through intentional or unintentional processes – has evolutionary consequences for the parasite. The location a zombie ant dies largely impacts the parasite's fitness, as the ant must die somewhere released spores will encounter and infect new ants. Preserving an ant's normal movement behavior might cause the ant to walk too far away from the nest, limiting transmission. Conversely, an excessive impact on ant movement might prevent the ant from leaving the nest (where the fungus cannot grow (Loreto et al., 2014)) or inhibit the ant's ability to climb up vegetation to a biting location. The different possibilities exemplify a tradeoff similar to that of virulence—a parasite with high virulence makes the host too sick to transmit, while a parasite with low virulence might not produce enough

infectious material for transmission (Frank, 1996; May & Anderson, 1983). Thus, parasites tend to evolve towards intermediate virulence, like how a zombie ant fungus has an intermediate impact on host movement.

In our model, some ant agents moved with a correlated random walk, as step length was fixed and turning was determined from a normal distribution with the mean as 0, indicating the individual will tend to persist in the same direction of travel (Turchin, 1998). However, when the standard deviation of the turning angles (the *turning-index*) is large, like for the simulated zombie ants, the walk resembles an uncorrelated random walk or Brownian motion. Interestingly, some ant trajectories have been shown to have properties of a random walks, particularly when engaged in search behavior (Crist & MacMahon, 1991; Torres-Contreras & Canals, 2010). For example, when a pair of ants is tandem running, removing the leader causes the follower ant to search in a way similar to Brownian motion (Nigel R. Franks et al., 2010). Such tortuous search paths can be inefficient, due to high path overlap, which explains why ants tend to have straighter trajectories at lower ant density (F. R. Adler & Gordon, 1992; Gordon, 1995) and ants better at discovering food resources move in straighter lines (Pearce-Duvet et al., 2011). However, zombie ants are not concerned with efficiency, only with the maximum allowable distance from the nest and foraging trails that allows the fungus to grow and release spores that future ants will encounter.

A circular, wandering walking style seems to achieve this—and other examples of infected animal movement support erratic movement as an infection outcome. Enhanced locomotory activity (ELA) refers to wandering and hyperactive behavior often exhibited by holometabolous insect larvae prior to pupation, thought to help the insect reach a suitable metamorphosis location (Nijhout, 1994). However, ELA can also be induced by baculovirus infection (Goulson, 1997). Other parasitized ants show differences in movement, such as how *Camponotus* ants infected with a liver fluke (*Dicrocoelium dendriticum*) were observed moving sluggishly, slowly circling rocks and sometimes remaining motionless (Carney, 1969).

Interestingly, our results indicate that movement cannot be overly impaired for ants to reach a biting location. For a high proportion of ants to reach a biting location, a higher turning rate must be matched with a larger step size (Fig. 3-3). This indicates the inefficiency of turning frequently as ants must walk faster in order to reach a biting location. When ants turn less, they must walk slower in order to stay within the maximum allowable distance where the fungus still has the potential to transmit to future ants. A caveat to the results is that step length was implemented as a constant in our model, meaning that zombie ants could instead be moving at variable speeds or even stopping or falling frequently, which has been observed in the field and laboratory (Hughes et al., 2011). Additionally, the rainforest patches surrounding the ant nests are typically dense with vegetation which was not captured in our simplified model space. Ants are likely walking up and down the vegetation in order to reach a biting spot, meaning turning is likely still frequent. An interesting follow-up study would investigate how adding complexity to the environment would alter zombie ant movement.

Overall, there are few mechanistic studies on how animal locomotion changes with infection status, perhaps because of limited techniques. Our work aims to show the power of combining field data with an agent-based modeling approach to investigate hypotheses of how infection alters animal behavior. We demonstrate that zombie ants likely turn more than uninfected ants, but the parasite does not need to control the ant to an exact location for the ant to bite in a position that appears adaptive for transmission. The results were robust to changes in time limit as well as consistent across nest set-ups. Moreover, this model sets the stage for future investigation on the adaptiveness of the biting locations, further opening up how we can approach questions on the proximate and ultimate causes of parasite manipulation.

Acknowledgements

We thank Dr. Raquel Loreto for the spatial data and original work on this system. This work was supported by the National Institutes of Health (R01 GM116927-02 to D.P.H).

Supplementary videos for chapter 3**:** https://doi.org/10.26207/3kky-0t33

Figures

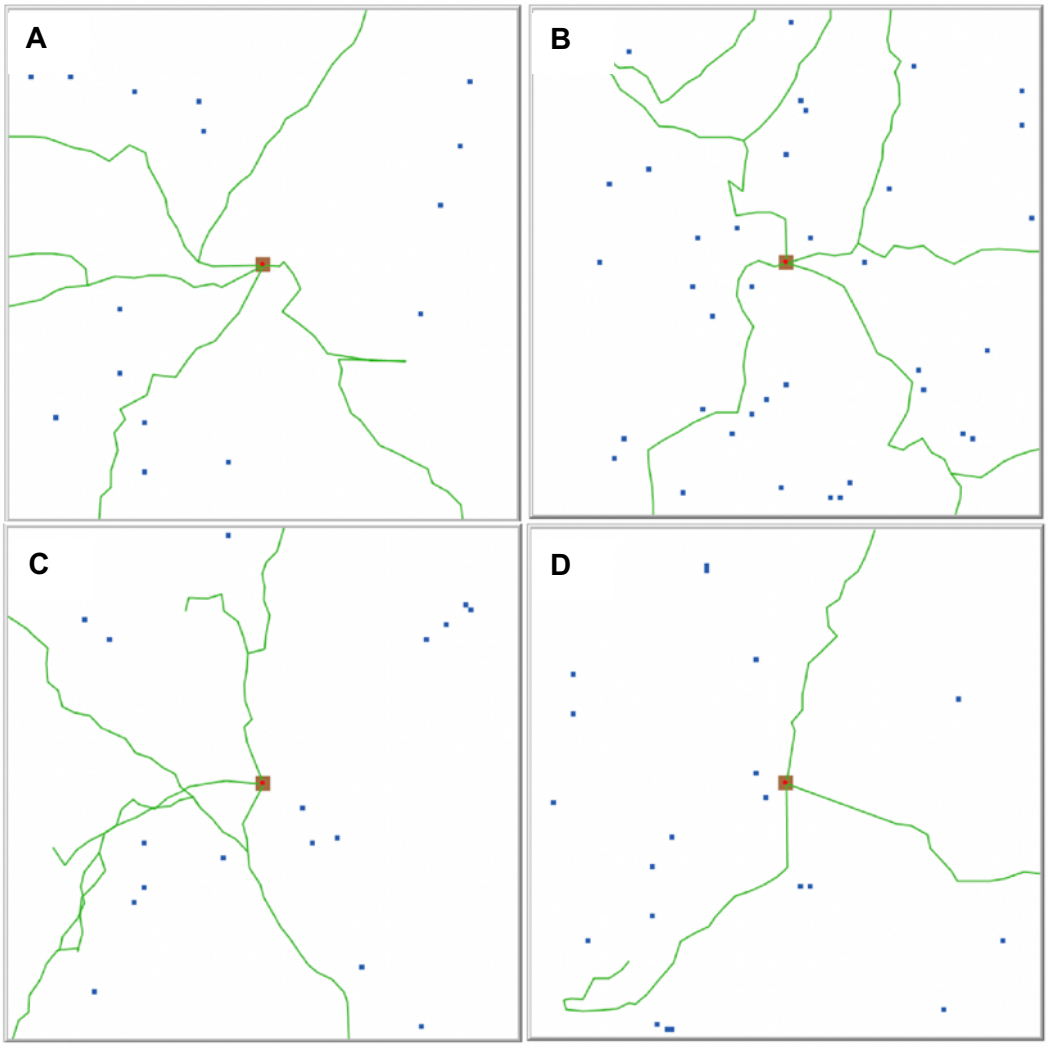


Figure 3-1. The location of the trails and zombie ant targets for model setups.

A) Nest 1 **B)** Nest 2 **C)** Nest 4 and **D)** Nest 5. Green lines represent the trails, blue squares are the zombie ant target locations, and the brown box is the nest. The size of the area replicated by the the model is 10m by 10m.

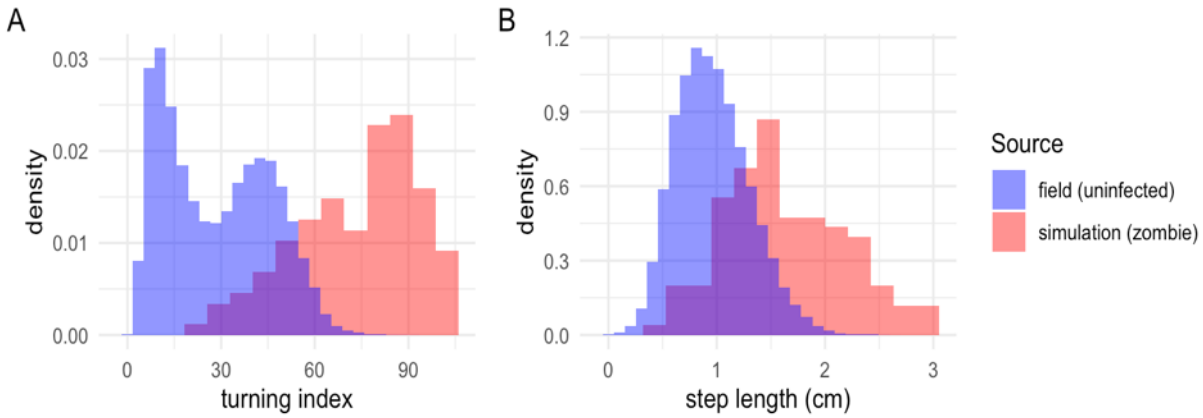


Figure 3-2. Comparison of simulated zombie ant movement values to ant movement values from the field.

Distributions of (A) turning index values and (B) step lengths obtained from simulations (red; n = 120) as compared to the field data of uninfected ant values (blue; n = 64,683). To standardize for the number of observations, we calculated the density across the range.

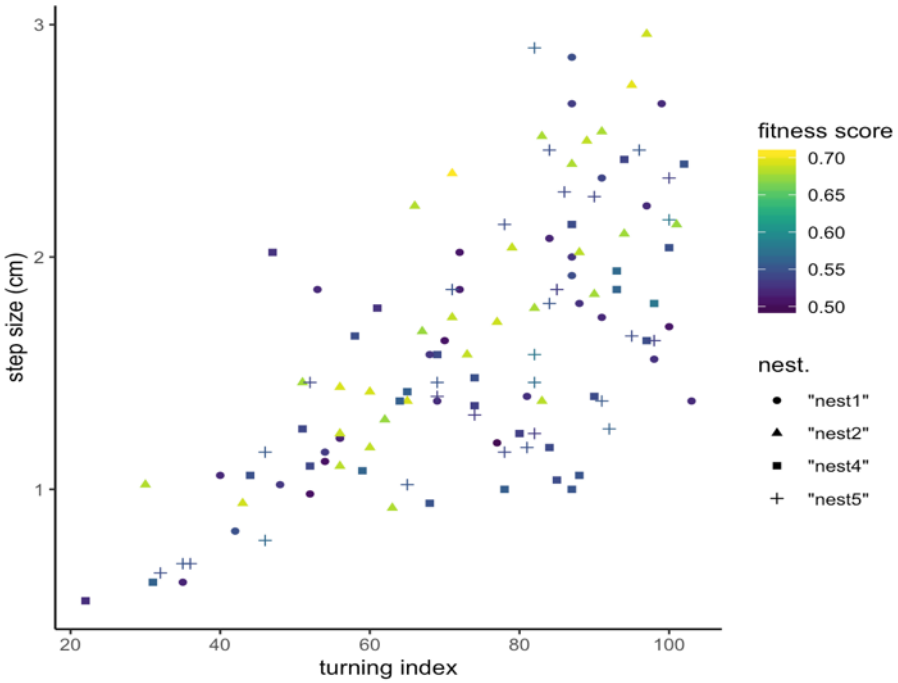


Figure 3-3. Parameter search results

Each point is the parameter combination that led to the highest fitness score (f) for a single parameter search (500 model runs). Higher turning rate was correlated with a larger step size. Each point shape indicates a different model setup and the point color indicates the maximize fitness score obtained from the indicated turning rate and step size.

Chapter 4

A new zombie ant behavior unraveled: aggregating on tree trunks[3]

Abstract

Hosts can be manipulated by parasites to move to locations advantageous for onward transmission. To investigate the role of behavioral manipulation in creating transmission hotspots, we studied the distribution of zombie turtle ants in the Amazon rainforest. The turtle ant *Cephalotes atratus* nests and mostly forages in the canopy, but is found at the base of trees when infected with the zombie ant fungus *Ophiocordyceps kniphofioides*. We found 626 infected cadavers on 14.7% of 162 trees sampled. Cadavers were highly aggregated on the surface of the trees, explained by behavioral observations indicating infected ants as slightly attracted to zombie ant cadavers on a tree. From 1,726 hours of camera footage, we recorded the removal of three zombie ant cadavers by live ants. The number of removals compared to the density of infected individuals indicates the base of a tree as an escape from the evolved ability of social insects to recognize and treat disease inside the nest, allowing the parasite to continuously remain in the environment.

[3] This chapter was published under the following citation:
Imirzian, N., Araújo, J.P. and Hughes, D.P., 2020. A new zombie ant behavior unraveled: Aggregating on tree trunks. *Journal of Invertebrate Pathology*, *177*, p.107499.

Introduction

Social insects form highly evolved societies (Wilson, 1971). The evolutionary pathway of social insects towards increased group living necessitated the evolution of functional traits that prevent the spread of disease through the colony (Cremer et al., 2007; Meunier, 2015). Much attention has been paid to the limited ability of parasites to persist in the nest (Stroeymeyt et al., 2014; Wilson-Rich et al., 2009), but less researched are the ways parasites can evade social insect defense behaviors (Araújo & Hughes, 2019; Hughes et al., 2008). Limited transmission within a colony may promote other routes of infection for social insects outside of the nest, such as parasitic manipulation of host behavior (Hughes, 2012).

Manipulative parasites can lead a host to an area that will heighten transmission and subsequently increase the parasite's fitness (Moore, 2002; Poulin, 2010). Striking examples include hosts drowning themselves to allow a parasitic worm to reproduce (F. Thomas et al., 2002) or rats losing their fear of cats when infected with *Toxoplasma gondii,* increasing the likelihood the rat will get eaten by the definitive cat host of the parasite (Berdoy et al. 2000). Parasites with direct life cycles, where transmission occurs directly from one host to the next without a free living stage or intermediate host, can also manipulate behavior, such as viruses that induce their host to spend increased time in a location where onward transmission is favored (Hoover et al., 2011). Unlike trophically transmitted parasites, parasites with direct life cycles cannot rely upon the predatory behavior of the next host and must place themselves where they are most likely to encounter their current host.

Directly transmitted parasites face unique challenges when infecting social insects. As central place foragers, most social insects return to the same nest after each foraging trip. Although the nest contains a high density of individuals, seemingly conducive for transmission,

colony organization and behavioral defense mechanisms limit insect-to-insect transmission within the nest (Cremer et al., 2007; Stroeymeyt et al., 2018). Social insects are known to remove corpses and diseased individuals (Baracchi et al., 2012; Diez et al., 2014; Renucci et al., 2011), reduce fungal spore loads through grooming (Okuno et al., 2012; Reber et al., 2011), as well as use antimicrobial compounds to prevent the growth of microorganisms (Brütsch et al., 2017; Graystock & Hughes, 2011; Tragust et al., 2013). However, returning to the same nest implies that workers move in similar areas of the environment, providing a predictable opportunity for parasites to infect susceptible foragers outside of the nest. If parasites are capable of manipulating the behavior of their host, it would be advantageous for the parasite to use host predictability to position a host where transmission is enhanced. Given that we know parasites aggregate within specific hosts in a population (Poulin, 1993; Shaw & Dobson, 1995; Wilson et al., 2002), our question is if parasites capable of manipulating their hosts can cause an aggregation of infectious individuals.

Host location is particularly important for parasites that produce propagules from an insect cadaver, like the baculovirus discussed above or certain fungal pathogens, since once the parasite kills the insect, the dispersal of infectious material is limited to a small area surrounding the cadaver. Some hypocrealean fungal entomopathogens, such as *Metarhizium* and *Beauveria*, produce huge numbers of small conidia that are passively transmitted (Hesketh et al., 2010; Humber, 2012). In contrast, some species-specific fungal pathogens, such as those in the Entomopthorales or *Ophiocordyceps* infecting ants, tend to produce fewer spores that are very large (Araújo & Hughes, 2017; Boomsma et al., 2014; Kobmoo et al., 2012). The location of the host cadaver for these species is especially important since the spores need to come in contact with a specific host. Interestingly, some of these fungi have evolved adaptations to increase transmission, such as manipulating a host to move to an elevated location to increase spore dispersal (Roy et al., 2006).

The fungal species complex *Ophiocordyceps unilateralis sensu latu* (Ascomycota, Hypocreales) became popularized as 'zombie ant fungi' for an ability to manipulate ants to leave the nest and die biting onto the underside of leaves or twigs (Andersen et al., 2009; Evans et al., 2011; Loreto et al., 2018). The zombie ant extended phenotype is characterized by this death grip or biting behavior, where the ant is attached to vegetation through its mandibles. We consider the infected ants as 'zombies' because the insect's behavior is altered in a way that benefits the parasite (Libersat et al., 2009; Roy et al., 2006). The majority of zombie ant studies have investigated the *O. unilateralis* group (currently comprised of 28 species), but fungi in the closely related *O. kniphofioides* complex (5 species, including *O. kniphofioides sensu stricto*) also manipulate ants and can be considered zombie ant fungi (Araújo et al., 2018).

Notable differences exist between these two fungal groups (Araújo & Hughes, 2017). Fungi in the *O. unilateralis* group mostly manipulate ants in the Camponotini tribe to die biting onto twigs or leaves (Loreto et al., 2018). In contrast, ants infected with fungi in the *O. kniphofioides* group tend to die biting into tree trunks, and are mostly Myrmicinae ants, as well as *Dolichoderus* and *Paraponera,* but never a Camponotini species (see Fig. 19 in Araújo et al. 2018). Additionally, the fungal structures produced by *O. kniphofioides sensu stricto* differ to the fungal structures produced by *O. unilateralis sensu latu.* Most commonly, *O. unilateralis s.l.* produces a single fungal stalk emerging from the ant's dorsal pronotum with an ascoma or sexual fruiting body (teleomorph) containing vermiform spores that are actively shot into the environment. The fungus also produces asexual morphs (anamorphs) with conidia transmitted by contact (*Hirsutella* anamorphs) (Araújo et al., 2018; Evans et al., 2011). Although *O. kniphofioides s.s.* can also produce a teleomorph, multiple types of anamorphs frequently grow from the infected ant cadavers (Fig. 4-1C-E; Araújo et al., 2018; Evans & Samson, 1982). The asexual conidia produced by *O. kniphofioides* are enveloped in mucus, forming a mucous droplet, which is a characteristic usually associated with transmission by contact (Fig. 4-1F-G; Evans &

Samson, 1982). Considering the risk of transmission by contact, we are interested in host behavior towards cadavers infected with *O. kniphofioides*.

The fungal species *O. kniphofioides sensu stricto* infects the turtle ant *Cephalotes atratus* and is particularly interesting due to a historical record documenting hundreds of ant cadavers found at the base of rainforest trees in the Brazilian Amazon (Evans & Samson, 1982). The ant host, *Cephalotes atratus*, is an arboreal ant widely distributed across the Neotropics (De Andrade & Urbani, 1999). Colonies can contain over 10,000 individuals spread among one or multiple nests in tree cavities of the same or nearby trees (Corn, 1976, 1980; Weber, 1957). Workers mainly forage in the canopy on hemipteran honeydew and extrafloral nectaries (Fig. 4-1A), but sometimes forage on the ground on arthropod remains and slow-moving prey (Corn, 1976). Nitrogen-recycling bacterial gut symbionts housed in specialized gut chambers support their herbivorous diet (Bution & Caetano, 2008; Hu et al., 2018; Russell et al., 2009), perhaps contributing to their abundance (Davidson et al., 2003). In turn, their abundance may have made them a target for specialized parasites, such as a nematode that turns the ant's abdomen red (Yanoviak et al., 2008) and a zombie ant fungus that is the focus of this study.

To determine the scale of zombie turtle ant aggregations, we first searched for hotspots of infection, or trees with large numbers of *C. atratus* ants infected with *O. kniphofioides* fungi. At each hotspot, we mapped out the spatial distributions of cadavers and determined the density of cadavers on the surface of the tree. We set up cameras to record the natural interactions between live *C. atratus* ants and the dead infected cadavers. We found that infected ants die in highly clumped distributions across multiple scales, as cadavers are found on specific trees in an area as well as clumped on the surface of a tree. Moreover, we observed live ants interacting with cadavers just before dying and biting, providing insight into the mechanism of our observations.

Methods

Spatial data

Research took place at Reserva Florestal Adolpho Ducke (02°55'S, 59°59W) near Manaus, Amazonas, Brazil, from 13 to 27 August 2018 and from 16 February to 9 March 2019. At the field site, we thoroughly inspected the base of trees for *Cephalotes atratus* cadavers killed by *Ophiocordyceps kniphofioides* (Fig. 4-1B-E). We searched for trees with a high density of cadavers. Trees with 10 or more cadavers were considered hotspots. We mapped out the trees surrounding a hotspot tree within a 10-meter radius and referred to the area as a 'graveyard' (Pontoppidan et al., 2009). We selected a distance of 10 meters since most ground foraging ants stay within this distance from the nest (Corn, 1976). We recorded the tree family, circumference at breast height, the number of cadavers, and distance and direction from the hotspot tree of all trees with a circumference above 20 centimeters. We focused on trees of this circumference since we have not found infected *C. atratus* cadavers on trees smaller than this size.

At each tree in a graveyard where we found *O. kniphofioides* infected *C. atratus* cadavers, we counted the number of cadavers and documented their location on the tree. We recorded the distance from the ground, degree of damage, type of fungal growth, and orientation on the tree. We recorded damage to cadavers as an estimate of age and how frequently other organisms interact with the infected cadavers. We measured damage on a 3-point scale: 0 = no damage, 1 = missing gaster (the abdomen of an ant), and 2 = missing gaster and thorax. We observed 3 morphologically distinct types of anamorphs growing from the cadavers, coinciding with the original fungal description (Evans & Samson, 1982). For clarification purposes, we called them type A (Fig. 4-1C), type B (Fig. 4-1D-E), and type C (close-up in Fig.4-1F-G, see also Fig. 22 in Araújo et al. 2018). Anamorph type A forms a fungal stalk that can then develop

into the sexual fruiting body or teleomorph. We recorded which anamorph was currently growing or if there was no fungal growth. Often, we found broken apart dead *C. atratus* ants at the base of the trees (see Fig. 23 in Araújo et al. 2018). We only counted heads with thoraxes at the base of trees as cadavers. We recorded the horizontal distance from the base of the tree for the cadavers found on the ground.

Field behavioral data

The cadavers with visible external fungal growth were filmed for 27 days (10 days from 13 August to 27 August and 17 days from 16 February to 9 March) to capture any interactions with conspecifics or other arthropods. We used GoPro cameras (model: HERO 5, GoPro, Inc., San Mateo, USA) to take a picture every 10 or 30-seconds at each focal cadaver then created a time-lapse video from these images to analyze these videos for interactions. Preliminary experiments determined a time-lapse interval of 30-seconds was sufficient to capture interactions, but improved storage capabilities allowed us to switch to taking an image every 10-seconds. In August, cadavers were only filmed during the day, while in February, cadavers were filmed for 24-hours a day. For night-time recording, we used infrared lights (IR30, CMVision, Houston, USA) as to not disturb behavior and GoPros with modified infrared filter (RageCams.com, Michigan, USA). We recorded at cadavers with anamorphs type 1 and 2, as well as at cadavers with the teleomorph.

We watched each time-lapse video to record if any invertebrate touched the cadaver or if a *C. atratus* ant walked in the background. A touch was defined as the invertebrate touching any part of the cadaver's body (including the legs) or walking over the surrounding fungal material. If a *C. atratus* ant touched a cadaver, we recorded the number of images where it touched the cadaver and multiplied the number of images by the time-lapse interval (either 2 or 6 frames per

minute) to estimate the total time the ant interacted with the cadaver. If the ant touched multiple cadavers located close together (within the same camera view), we considered this as one interaction event and added the time spent touching either cadaver together.

Statistical methods

We used linear regression to test whether larger trees have more cadavers, using tree circumference as the explanatory variable and cadaver density (number of cadavers/m^2) as the response variable. To determine the density of cadavers, the number of cadavers on a tree was divided by the surface area of the tree. The tree circumference at breast height and a height of 2.5 meters (the highest a cadaver was found was 2.2 meters high) was used to calculate the surface area for all trees. Next, we investigated how aggregated cadavers are on the trees with 10 or more cadavers using Monte-Carlo simulations. A number of points equal to the number of cadavers found on a tree was randomly distributed in an area the same size of the tree sampled. The mean nearest neighbor distance was calculated for 500 simulated distributions of points for each tree. To get a p-value, we calculated the number of times the actual mean nearest neighbor value was greater than the simulated mean nearest neighbor value and divided that by the number of simulations. All analyses were performed in RStudio version 1.1.463 (RStudio Team, 2016) and R version 3.4.4 (R Core Team, 2018).

Results

Spatial distribution

We found a total of 626 *Cephalotes atratus* ant cadavers infected with the fungus *Ophiocordyceps kniphofioides* across 6 graveyards in a patch of Brazilian Amazon rainforest. All cadavers counted were assumed to be infected. This assumption is valid since biting behavior is only recorded from infected ants. The sample areas of the 6 graveyards had a total of 162 trees and 24 of those trees (14.8%) had cadavers (Fig. 4-2). The number of infected cadavers found in a graveyard ranged from 20 to 246 ants (median: 79 ants; G1 = 176, G2 = 20, G3 = 29, G4 = 246, G5 = 129, G6 = 26). Cadavers usually concentrated on one to three trees in the sample area (Fig. 4-2). An average of 85.2% of trees did not have cadavers in a graveyard (G1: 25/27, 92.6%; G2: 21/23, 91.3%; G3: 23/28, 82.1%; G4: 26/32, 81.5%; G5: 23/27, 85.2%; G6: 21/25, 84.0%).

Larger trees did not have a higher density of cadavers (linear regression: $F_{1,21}= 1.25$, $p = 0.28$). Zombie ants were found on trees from 11 different families (Figure AC-1). The most common tree families found with cadavers were Fabaceae and Sapotaceae (63.6% and 36.4% of trees with cadavers, respectively). However, tree families did not significantly differ in the proportion of trees found with an infected cadaver (Fisher's Exact Test; $p = 0.22$).

Most cadavers clustered around the base of the trees (Fig. 4-3). The mean cadaver height above the ground ± the standard deviation was 30.64 ± 43.39 centimeters. At each tree with a concentration of cadavers greater than 20, zombie ant cadavers are located significantly closer together than expected from complete spatial randomness (Table AC-1; Monte-Carlo simulations; $p < 0.001$). For trees with less than 20 cadavers, cadavers were not more clumped than expected from complete spatial randomness (Table AC-1; Monte-Carlo simulations; $p > 0.3$). A majority of cadavers were undamaged (70.4%, n = 441), but 25.7% (n = 161) were missing a gaster

(abdomen), and 2.72% (n = 17) were missing a gaster and a thorax. Seven cadavers were knocked from the tree and lost in the leaf litter before we could determine the degree of damage.

The majority of cadavers (69.8%; n = 442) did not yet show external fungal growth, though some showed growth beginning in the ant's joints (Fig. 4-1E). Only 5.21% (n = 33) of cadavers were growing fungal anamorph type A, some with a mature teleomorph while others with only the stalk (Fig. 4-1B). In contrast, 11.1% (n = 70) were growing fungal anamorph type B (Fig. 4-1D) and 14.4% (n = 91) were growing fungal anamorph type C (Fig. 4-1F-G). The graveyards differed in the most common type of anamorph. In graveyards 1, 3, and 5, type B was the most common anamorph growing, at 94.9% (n = 37), 60% (n = 12), and 100% (n = 15) of cadavers growing fungi, respectively. In comparison, fungal anamorph type C was most common in graveyards 2, 4, and 6 as it was present on 50% (n = 2), 73.5% (n = 72), and 73.3% (n = 11) of cadavers growing fungi.

Field behavior

We recorded 22 ant cadavers across 27 days, capturing a total of 418,406 time-lapse pictures amounting to 1,726.63 hours of film coverage (Table 4-1). During this time, we observed an invertebrate touching a cadaver 108 times. Out of those 108 cadaver interactions, 11 cadaver touches were by a *Cephalotes atratus* ant. Additionally, *C. atratus* ants were observed in the videos but not touching a cadaver 14 times. During 3 of the *C. atratus* cadaver interactions, the cadaver was removed from the tree by the *C. atratus* ants (Supplementary Video S4-1). Removal occurred twice at night and once during the daytime. Removal took 19.17 minutes, 21.33 minutes, and 100.67 minutes. In the removal taking over an hour, the cadaver was removed by two ants working together (Removal Event 1 – Supp. Video S4-1).

In the 8 remaining *C. atratus* interactions, the ants touched but did not remove the cadaver (Supplementary Video S4-2). On average, *C. atratus* ants touched a cadaver for 8.21 minutes, with a range from 1 minute to 19.5 minutes. Combining removal and touch events, 11 *C. atratus* ants interacted with cadavers for a total of 206.83 minutes or 3.44 hours, amounting to 0.19% of the total video footage.

We additionally observed 5 infected ants immediately before biting into the tree and dying. The main activities performed by the ants before biting were pulling at moss, boring into the tree bark, and twitching (Supplementary Video S4-1). Many of the observed cadavers died burrowed into the tree bark (Fig. 4-1B). We observed an ant picking the fungal material off a cadaver on a tree in graveyard one (Zombie 1 - Supplementary Video S4-1). Shortly afterwards the ant bit into the tree trunk below the cadaver (Supplementary Video S4-1). Of the five fresh cadavers observed, three were removed during the study period. The first was removed in 1 day, the second in 10 days, and the third in 4 days (average number of days until removal = 5 days). The cadaver that was removed in 4 days (Zombie 5 - Supp. Video S4-3) was caught on camera being removed (Removal Event 3 - Supp. Video S4-1).

Discussion

We found *Ophiocordyceps kniphofioides* infected *Cephalotes atratus* ants die in highly aggregated distributions across multiple scales. Only 14.8% of trees sampled had cadavers, indicating that behavioral manipulation of ants by this fungus leads infected ants to preferentially occur on certain trees in an area (Fig. 4-2). Manipulation by this fungus also includes directed behavior on a tree as cadavers were clumped together near the bottom of trees (Fig. 4-3). Some live ants, which were suspected but could not be confirmed as infected, showed signs of attraction to the cadavers (Supp. Video S4-2 and 3) We recorded five manipulated ants just before biting,

three cadavers being removed by *C. atratus* ants, and eight *C. atratus* ants touching cadavers. Ants tend to die biting into moss or bark with the fungus growing under the bark, which might allow persistence of fungal structures in the environment even after the ant host cadaver is removed (Fig. 4-1B, D; Supp. Video S4-1). The low number of interactions (only 0.19% of the 1,726 total hours of coverage on 4 trees over 27 days) suggests that epidemics are not a major feature of this system and infection is maintained with a low frequency of transmission.

Only 26 *C. atratus* ants were observed on the tree trunks in the 1,726 hours of video coverage (Table 4-1), contributing to the low number of interactions. Similarly, in Thailand, the carpenter ant *Camponotus leonardi* was rarely observed outside of the canopy but found in dense aggregations near the forest floor when infected with *Ophiocordyceps unilateralis s.l.* (Pontoppidan et al., 2009). The similarity between systems, despite different continents, host and parasite species, indicates this as part of an evolved parasitic strategy to infect ants. Unlike manipulative parasites that have free-living stages (e.g., hairworms that induce terrestrial insects to enter water (Thomas et al. 2002)) or manipulative parasites which exploit already well-adapted systems of predation (i.e., trophic transmission: Carney 1969; Berdoy et al. 2000; Cezilly et al. 2000; Yanoviak et al. 2008), this fungal parasite must find new hosts while limited by the distinctive behavioral ecology of its current host. Principally, effective transmission must contend with the anti-parasite defense behaviors of the host (Cremer et al., 2007), which likely limits the parasite's ability to transmit to other hosts inside of the ant nest. One known mechanism to overcome within nest defenses is inducing infected insects to leave the colony so onward transmission to foraging workers can occur (Araújo & Hughes, 2019; Hughes & Libersat, 2019; Loreto et al., 2014).

Manipulating ants to leave the nest heightens the importance of where infected ants die as they must encounter future hosts to transmit. After ants are infected by *Ophiocordyceps sp.*, the fungus grows within the ant for approximately 2-3 weeks (de Bekker et al., 2015; Sakolrak,

Blatrix, Sangwanit, & Kobmoo, 2018), while remaining undetected by nestmates within the colony (Gracia et al., 2018). The fungus is unable to grow within the nest (Loreto et al., 2014), meaning the parasite's fitness relies on the infected ant leaving and dying outside of the nest. Location is particularly important for *O. kniphofioides*, since only 5.21% of cadavers found were growing the fungal structure that leads to the teleomorph that can distribute spores through the air. In contrast, the more common fungal structures growing on 25.2% of the cadavers likely require direct contact by a new host for transmission. Thus, cadavers must die where future hosts will encounter them, perhaps leading the parasite to direct infected hosts to specific trees in the environment (Fig. 4-2).

The hotspot trees could not be explained by the tree family or the size of the tree. Instead, the ecology of the host might explain why the cadavers aggregated on certain trees in the area. The trees with cadavers might reflect the location of a nest, or where foraging ants come down from the canopy. This ant species exhibits an anti-predator defense behavior where workers jump from aerial walkways to descend in a directed manner using gliding to alight on tree bases (Yanoviak et al., 2005, 2010; Yanoviak & Dudley, 2006). One hypothesis is that certain trees are landed on and climbed more frequently following such descents, promoting the aggregation of cadavers on certain trees in the environment (Fig. 4-2). A future study documenting the movement of ants from the canopy would be useful for investigating the full transmission cycle of the system.

However, host ecology does not explain why cadavers were highly aggregated on the surface of target trees (Fig. 4-3), sometimes even biting on top of each other (Fig. 4-1E). Another hypothesis is that the aggregation is due to an autocatalytic mechanism emerging from interactions between live infected ants and cadavers. Entomopathogenic fungi are known to lure insects using chemical mimicry (Roy et al., 2006). The parasite could benefit from an infected ant recognizing other infected cadavers since it could indicate an area with a suitable density of hosts

for transmission. Interestingly, our results imply that there is a threshold needed for aggregation to begin, as cadavers were not more aggregated on a tree than expected from a random distribution unless there were more than 20 cadavers on a tree (Table AC-1). Simple individual rules can drive collective patterns, such as an increased stopping rate near an aggregation (Ame et al., 2004; Deneubourg & Goss, 1989). Our video observations showing zombie ants interacting with cadavers just prior to biting (Supp. Video S4-3) indicate attraction could be playing a role in creating the zombie ant aggregations.

While infected ants might be lured by zombie ant cadaver volatiles, we suggest a different interaction between uninfected ants and infected cadavers. Interestingly, the cadavers removed did not have extensive external fungal growth (Supp. Video S4-1), in addition to the cadavers that were touched (Supp. Video S4-2). Ants are known to modify their behavior depending on fungal concentration (Konrad et al., 2018; Okuno et al., 2012; Pereira & Detrain, 2020), implying that the uninfected ants might avoid heavily sporulating cadavers. Ants avoiding highly infective cadavers could explain the low number of *C. atratus* interactions observed in our study (Table 4-1).

Yet, foraging *C. atratus* ants were rarely observed on the graveyard trees, indicating that the low number of interactions could be due to ants infrequently coming down from the canopy. The low encounter rate might protect the fungus from excessive removal behavior, allowing the fungus to remain on hotspot trees throughout the entire year (Evans & Samson, 1982). Meanwhile, producing fungal structures that extend out from the cadaver and remain on the tree substrate (Fig. 4-1D) could create infection points for unsuspecting ants even in the absence of cadavers. It would be useful to study the interaction between uninfected ants and the different fungal anamorphs growing with and without a cadaver to elucidate whether avoidance, attraction, or no reaction is occurring, and whether that behavior is modified by the presence of a cadaver.

In conclusion, manipulating infected ants to solidly bite into tree bark, away from a high traffic foraging area, may limit the host's anti-parasite defense behaviors and allows the parasite to use tree trunks as transmission hubs. A rule that relies on a threshold of infected cadavers to begin aggregating creates a situation where fungal infected cadavers group in an area with sufficient host density to improve the chances of transmission. At the same time, being distant from the main host encounter areas leads to a low frequency of transmission, lessening the chances of an epizootic and promoting coexistence of host and parasite.

Acknowledgements

We are grateful to Antônio Tavares Mello for identifying the tree families and assisting with field work. We also thank the Instituto Nacional de Pesquisas da Amazônia (INPA) for permission to work at the field site. This work was supported by a Grant-In-Aid of Research from Sigma Xi awarded to N.I., National Science Foundation Grants IOS-1558062 and EEID 1414296 to D.P.H, and NIH Grant R01 GM116927-02 to D.P.H.

Supplementary videos for chapter 4: https://doi.org/10.26207/5py3-jr36

Tables

Table 4-1. Zombie ant cadaver interactions.

Total amount of footage captured and the number of interactions with *Ophiocordyceps kniphofioides* infected *Cephalotes atratus* cadavers on tree trunks.

	August	Feb/March	Total
Number of days cameras set-up	10	17	27
Hours recorded	422.65	1303.98	1726.63
Number of cadaver interactions	14	94	108
Number of *C. atratus* cadaver interactions	1	11	12
Avg. time *C. atratus* spent touching a cadaver (minutes)	19.5	7.52	8.21
Number of times *C. atratus* in camera view	7	19	26
Number of cadavers removed	0	3	3
Number of zombie ants observed biting	0	5	5

Figures

Figure 4-1. Images of uninfected and infected *Cephalotes atratus* ants.

(A) Uninfected foraging *Cephalotes atratus* ants tending honeydew-producing treehoppers (one marked with a red arrow -- difficult to see since their color, size and texture closely match that of *C. atratus*) **(B)** Two recently killed ants biting into crevices on the tree trunk **(C)** *C. atratus* infected with *Ophiocordyceps kniphofioides* (zombie turtle ants) growing anamorph type-A, that will later become the sexual morph (**D)** Anamorph type-B (*Hirsutella*-like) growing around *C. atratus* cadaver (**E)** Two infected ants dead next to each other. Note the white cottony structures arising from abdomen and leg joints, evidencing early stages of fungal growth. **(F)** and **(G)** Close up of mucous droplet containing conidia formed from anamorph type C. Credits: **A)** Alex Wild; **B–F)** João Araújo.

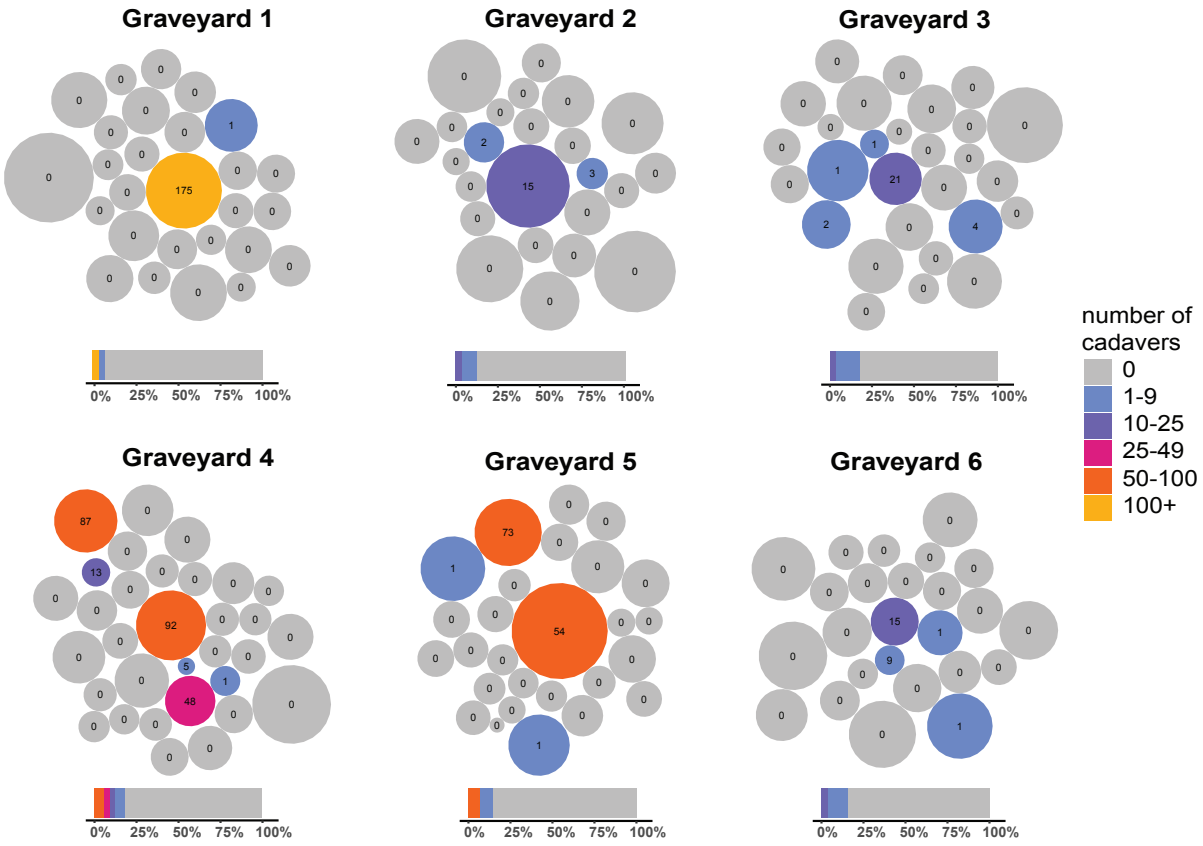


Figure 4-2. The number of infected cadavers on each graveyard tree.

Circles represent individual trees and size is scaled to tree circumference. Center numbers indicate how many Ophiocordyceps kniphofioides infected Cephalotes atratus cadavers were found on that tree. Circle locations correspond to the direction from the hotspot/center tree (see the uncondensed layout in Fig. AC-2). The bar below each graveyard shows the percent of trees with the number of cadavers indicated by the scale bar.

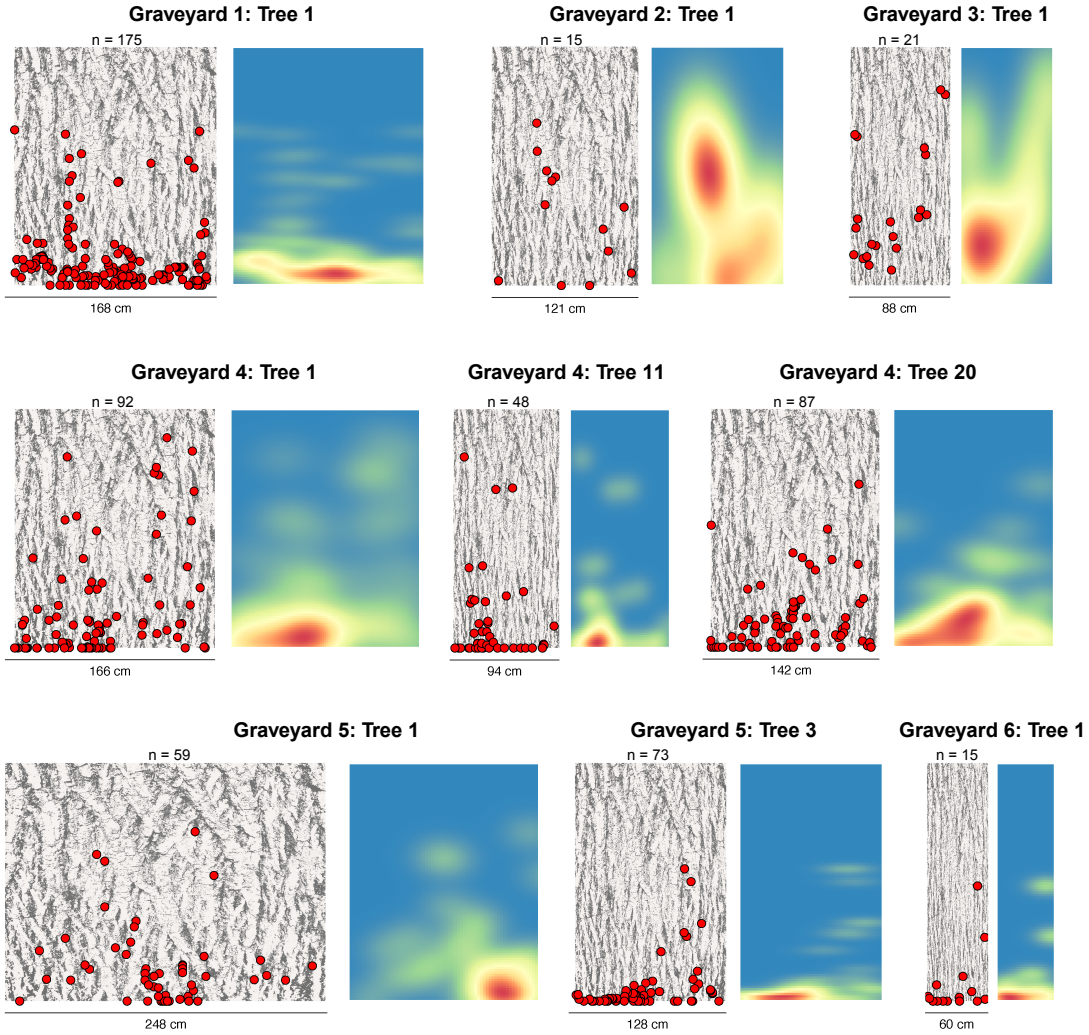


Figure 4-3. Location of zombie ant cadavers on the surface of the trees.

The red points indicate where a cadaver was found on the surface of the tree. The horizontal distance of each point is the curved distance from the north-facing side of the tree, and the vertical distance is the height above the ground for each point. The horizontal bar below each plot indicates the circumference at breast height of each tree. The total height represented for each tree is 2.5 meters. The heatmap to the right of each tree surface represents the density of the cadavers found on the trees, with blue representing the lowest density and red representing the highest density. Only trees with 15 or more cadavers were included

Chapter 5

Removal of zombie ant cadavers by conspecifics: social insect strategy limiting parasite diversification?

Abstract

The ants (Hymenoptera: Formicidae) form one of the most abundant groups of animals on earth. Part of their success has been attributed to an ability to defend the nest against disease. However, certain specialized parasites of ants, such as fungi in the *Ophiocordyceps* genus (Ascomycota, Hypocreales), have evolved to manipulate ants to die outside of the nest possibly to avoid colony defense behaviors. We investigated *Ophiocordyceps oecophyllae* infecting green tree ants (*Oecophylla smaragdina*), which is unique within the phylogeny for several reasons: (1) the infected cadavers are always found damaged, (2) the fungus grows from the ant cadaver differently than other *Ophiocordyceps* species and (3) it appears to be a dead-end species in the parasite phylogeny. Through behavioral observations, we demonstrate a mechanism for the damage to the cadavers by *O. smaragdina* ants. Thus, despite being a highly specialized manipulative parasite, the fungus appears to be limited by host behavior.

Introduction

Parasites and hosts are in opposition to each other. While the parasite aims to evade detection by the host, the host benefits from recognizing and destroying the parasite. Some parasites have evolved clever ways of overcoming host defenses and enhancing transmission through manipulation of host behavior (Poulin, 2010). Manipulative parasites can lead hosts to new environments where transmission is enhanced (Ponton et al., 2011; Wesołowska & Wesołowski, 2014), make hosts less fearful of predators that are the definitive host of the parasite (Baldauf et al., 2007; Berdoy et al., 2000), or alter social behaviors to increase interactions with new hosts (Burand et al., 2005; Keesey et al., 2017).

Zombie ants, which are ants manipulated by fungi in the genus *Ophiocordyceps* to leave the nest and die biting onto vegetation, are a popular example of parasite manipulation (Andersen et al., 2009). Manipulating the ant host to leave the nest is thought to help the parasite evade the defense behaviors of ants within the nest, as social insects have a variety of anti-parasite defense behaviors known as social immunity (Cremer et al., 2007). This has been a successful strategy for some parasitic fungi in the genus, as large aggregations of infected ants, known as 'graveyards', can be found in the environment (Chapter 4, Loreto et al., 2014; Pontoppidan et al., 2009). Fungi forming graveyards are typically within the *O. unilateralis sensu latu* group (Fig. 5-1A). Fungi within this group produce a fungal stalk that grows out of the ant's head with a spore producing fruiting body called an ascoma (Fig. 5-1C). Producing such a fruiting body allows distribution of spores through the air to the surrounding environment, allowing transmission to new ants without the ant touching the infected cadaver, and potentially allowing distribution onto high encounter areas such as a foraging trails (Loreto et al., 2014).

However, in contrast to other *Ophiocordyceps* species, ants infected with the parasite *Ophiocordyceps oecophyllae* do not produce a fungal stalk and have only ever been found damaged (Fig. 5-1B). The host species for *O. oecophyllae* is the green ant *Oecophylla smaragdina* (Araújo et al., 2018), which is abundant through its range from southern Asia to north-eastern Australia (Wetterer, 2017). The fungal growth of *O. oecophyllae* is limited to asexual fungal structures emerging from the leg joints and fissures, which suggests transmission requires a new host to touch the infected cadaver (Fig. 5-1B; Araújo et al. 2018). Considering the different transmission modes, one would expect species in the *O. unilateralis* group to more easily infect ants than *O. oeceophyllae*, since spores are released to a larger area from *O. unilateralis* fungi.

Indeed, the *Ophiocordyceps unilateralis sensu latu* species complex has 28 described species, with likely more to be discovered (Evans, Elliot, and Hughes 2011), while the group containing *Ophiocordyceps oecophyllae* has only 1 described species (Fig 5-1; Araújo et al. 2018). Thus, in some parts of the *Ophiocordyceps* lineage, the parasite's strategy appears to have facilitated diversification, while *Ophiocordyceps oecophyllae* seems a dead-end species. Green ants are well documented for their aggression towards other colonies and arthropods (Holldobler 1983; Peng, Christian, and Gibb 1995, 1997; Crozier et al. 2010), as well as their ability to control over 40 species of insect pests on many tropical tree crops (Way and Khoo 1992). Given the noted aggressive behavior of the host, we hypothesize that uninfected green ants are causing the damage to *O. oecophyllae* infected cadavers.

In this study, we set out to test our hypothesis and solve the mystery of why *Ophiocordyceps oecophyllae* infected cadavers are always found damaged. We first performed surveys to assess the abundance of the parasite, seeing whether the host is commonly infected and the fungus is prevalent in the environment. We then set up cameras and continuously recorded *O.*

oecophyllae infected cadavers to determine the cause of damage. Overall, we found that while the host species is abundant, the parasite is rare, likely due to the aggressiveness of the host.

Methods

Surveying for infected ants

In October 2018 and April 2019, we searched for *Oecophylla smaragdina* ants infected with the zombie ant fungus *Ophiocordyceps oecophyllae* from the Daintree National Rainforest (Cape Tribulation, Queensland, Australia) in the North to Djiru National Park in the South (Djiru, Queensland, Australia. Infected ants could be identified by a characteristic behavior of biting onto the underside of a leaf (Fig. 5-1), not known to occur with uninfected ants. To find infected *O. smaragdina*, we looked beneath the leaves of all of the trees in an area, focusing more intently on trees near *O. smaragdina* nests or large foraging trails. When an infected ant was found, we recorded the height above the ground, the location on the leaf, and the level of damage to the cadaver: legs missing, gaster (ant abdomen) missing, or gaster and thorax missing.

We combined our data with previous unpublished survey data from July 2011. During this survey, the infected ant cadavers were collected and pinned. A macro photograph was taken of each pinned specimen to determine how the cadavers were angled against the leaf they were biting. The angle of the ant against the leaf was measured using the Fiji distribution of the software program ImageJ (Schindelin et al., 2012). We measured the angle formed between the surface of the leaf and the body of the thorax. We were unable to measure the angle for damaged ants with only the head remaining.

Behavioral observations

To assess the cause of damage to infected *O. smaragdina* ants, we filmed infected ant cadavers using GoPro cameras (model: HERO 5, GoPro, Inc., San Mateo, USA). Filming occurred from 24 April to 3 May 2019 on the property of the Daintree Rainforest Observatory (James Cook University, Cape Tribulation, Queensland, Australia). Cadavers were filmed for 24-hours a day for up to 1 week or until the ant was removed. For night filming, we used cameras with a modified infrared filter (RageCams.com, Michigan, USA) and illuminated the cadaver with a small infrared light (IR30, CMVision, Houston, USA) connected to a 12-Volt 7Ah batteries (PS 1270S, Century Yuasa Batteries, Carole Park, Queensland, Australia). The GoPro cameras took pictures every 10-seconds and converted these images into time-lapse videos. We watched each time-lapse video and recorded if any organism touched or damaged the cadavers.

Results

Survey results

From 11 October to 28 November 2019, we found 1 infected *O. smaragdina*. From 19 April to 28 April 2020, we found 6 infected *O. smaragdina*. We additionally examined 20 pinned specimens of infected *O. smaragdina* ants that were collected in July 2011.

Manipulation attributes

All *O. smaragdina* cadavers found (n = 39) were missing a gaster (Fig. 5-2A). Only the head was remaining for 13 out of 39 (33.3%) of the cadavers found. Sometimes the legs and antennae would remain (Fig. 5-3A-B) or sometimes the legs and antennae would be completely

removed (Fig. 5-3C-D). Ants were angled an average of 46.7 ± 21.6° away from the leaf surface (Fig. 5-2B). and attached to leaves an average of 143.6 ± 21.8 cm above the ground (Fig. 5-2C).

Behavioral observations

We filmed a total of 5 *O. smaragdina* cadavers, 2 of which were completely removed during the filming period (Supplementary Videos S5-1 and S5-2). The first ant that was removed still had its legs attached when it was found (Fig. 5-3A). The legs were removed by an unidentified Orthopteran the same night as the cadaver was found (Supplementary Video S5-3). The next day the remaining parts of the ant were removed by an *O. smaragdina* ant in approximately 20 minutes (Supplementary Video S5-2). The second cadaver that was removed was pulled apart over a period of 3 hours by an *O. smaragdina* ant (Supplementary Video S5-1). Additionally, another cadaver that was found with its legs remaining (Fig. 5-3B) was removed within 3 days of discovery, but this was not caught on camera. No interactions were recorded with the other 3 cadavers during the filming period, apart from a spider touching a cadaver briefly. The cadavers that remained had an older looking appearance and more visible fungal material (Fig. 5-3C-E).

Discussion

In this study, we determined that infected green tree ant cadavers are found damaged due to host behavior. Green tree ants (*Oecophylla smaragdina*) parasitized by the manipulative fungus *Ophiocordyceps oecophyllae* were removed by conspecifics in the area, eliminating the infectious material that could infect new ants. Interestingly, all removed ants appeared newly infected, while older cadavers with visible fungal material were not touched (Fig. 5-3). We

suggest that ants modulate their behavior depending on the perceived risk of the fungal material. This aggression towards infected cadavers, limiting the number of parasitized ants in the area, could explain the lack of speciation in this lineage when compared to other ant parasitic fungi in the *Ophiocordyceps* genus (Fig. 5-1).

However, an opposite possibility is that the parasite induces the observed removal behavior in order to enhance its transmission. The morphology of *O. oecophyllae* suggests that an ant must touch the cadaver for transmission (Araújo et al., 2018). Thus, it would be advantageous for *O. oecophyllae* to evolve to attract the ant host, but we would expect to see live ants interacting with cadavers in the video footage if attraction was occurring. Apart from the cadavers that were removed, we observed no green ants touching cadavers in the continuous recording of cadavers over 6 days. Furthermore, the low number of parasitized cadavers found in our study (over 200 hours of searching uncovered 7 cadavers), indicate that if host attraction is occurring it is an ineffective strategy. Especially considering cadavers were typically located near *O. smaragdina* nests and foraging trails.

A question remains regarding why some cadavers persisted in the environment while others were removed within a day of discovery. Social insects are known to modify their behavior depending on the perceived infection risk. Ants are less likely to touch fungal infected cadavers that are sporulating (Pereira & Detrain, 2020) and will modify their grooming behavior depending on fungal concentration (Konrad et al., 2018; Okuno et al., 2012). Perhaps, in our study, foraging ants were able to perceive the higher concentration of spores on the older cadavers and consequently avoid those cadavers. A similar pattern was observed for a distantly related Entomophthorales fungal parasite, *Pandora formicae* infecting *Formica rufa* ants, that also manipulates ants to die biting into vegetation (Małagocka et al., 2017). Worker ants removed infected ants from the stems of grass surrounding a colony, but only if there was not extensive fungal growth (Marikovsky, 1962).

Infected cadaver removal by conspecifics also occurs for a different species of zombie ant, *Ophiocordyceps kniphofioides* infecting the ant *Cephalotes atratus* (Fig. 5-1D; Chapter 4, Evans and Samson 1982). However, in contrast to *O. oecophyllae,* large numbers of *O. kniphofioides* infected cadavers are still found in the environment despite removal, likely because the fungus remains growing on the substrate after removal (Fig. 5-1D; Evans and Samson 1982; Araújo et al. 2018). In fact, dense aggregations of infected ants known as 'graveyards' can be found for both zombie ants in both the *O. kniphofioides* and *O. unilateralis* group (Pontoppidan et al. 2009; Loreto et al. 2014; Evans and Samson 1982). No graveyards have been observed for *O. oecophyllae* infected ants, perhaps due to differences in fungal morphology, as *O. oecophyllae* lacks the fruiting body observed in *O. unilateralis* fungi (Fig. 5-1C) or an ability to grow onto the substrate as seen with *O. kniphofioides* (Fig. 5-1D). A study comparing transmission rates for the different fungal types would be useful to elucidate whether fungal morphology is a factor limiting *O. oecophyllae* infection.

Currently, the evidence suggests host behavior plays a role in limiting *O. oecophyllae*, as all cadavers were found damaged (Fig. 5-2). It is important to note that the host may not be causing all of the damage to the cadavers. We observed an unidentified Orthopteran removing the legs from one of the cadavers (Supp. Video S5-3). Interestingly, the insect did not fully remove the cadaver, only the legs. The cadavers might be difficult to remove, as shown by Supplementary Video S1 where the ant was observed pulling on the cadaver for over an hour. Similarly, in the removal of *P. formicae* infected ants, the ants exerted much effort when pulling off the cadavers and often removed them in pieces (Marikovsky, 1962). The strength required for removal may explain why we found cadavers missing parts of their body, instead of the whole cadaver removed, as some insects or ants may not be able to remove the whole cadaver.

Overall, this work explores how host behavior might explain the status of *O. oeceophyllae* as a dead-end species in a group showing high levels of diversification. The host

may limit persistence of the parasite in the environment by removing cadavers, reducing opportunities for the parasite to adapt and evolve. Coupled with the limited dispersal of the parasite's spores, we provide an explanation for why this parasite is comparatively rare. The *Ophiocordyceps* genus thus provides a good system to investigate how the outcome of specialization is varied, sometimes increasing and sometimes decreasing diversity, and should not be considered a universal factor that drives evolution in one direction. Moreover, it shows the incredible ability of ants to fight back against threats to their colony, explaining their status as a diverse and abundant group.

Acknowledgements

We are grateful for permission to work at James Cook University's Daintree Rainforest Observatory research station and the station staff for their help. We also thank Anna Schmidt for the preliminary work on the system and Dr. Sandra Abell for logistical support. This work was supported in part by a NIH Grant R01 GM116927-02 to D.P.H.

Supplementary videos for chapter 5**:** https://doi.org/10.26207/2e0r-az58

Figures

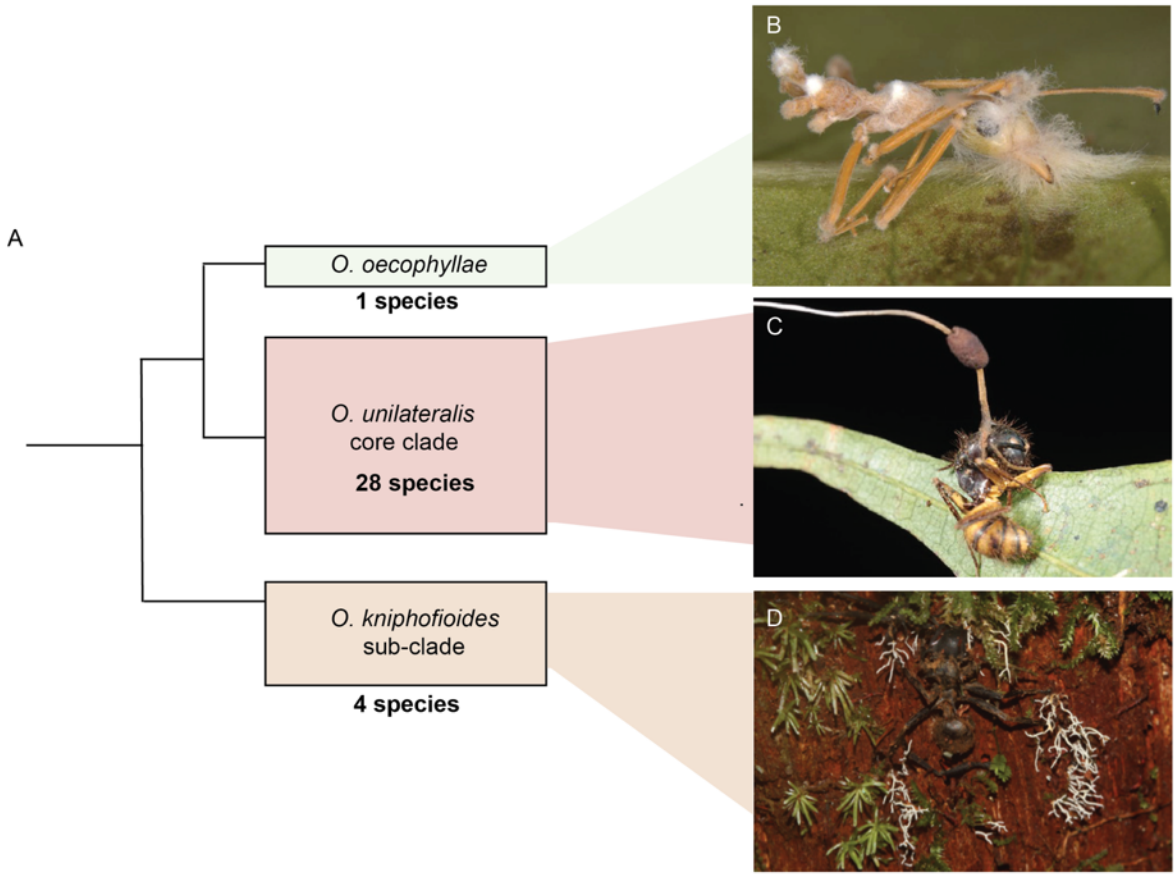


Figure 5-1. Simplified phylogeny of the ant parasitic fungi in the *Ophiocordyceps* genus. Each fungal species is only known to parasitize one ant species.

A) Phylogeny summarized from Araújo et al. (2018). **B)** *Ophiocordyceps oecophyllae* parasitizing the ant *Oecophylla smaragdina* **C)** *Ophiocordyceps camponoti-atricipis* parasitzing the ant *Camponotus atriceps*, with fungal stalk and ascoma emerging from ant **D)** *Ophiocordyceps kniphofioides* infecting the ant *Cephalotes atratus* with white fungal material surrounding ant.

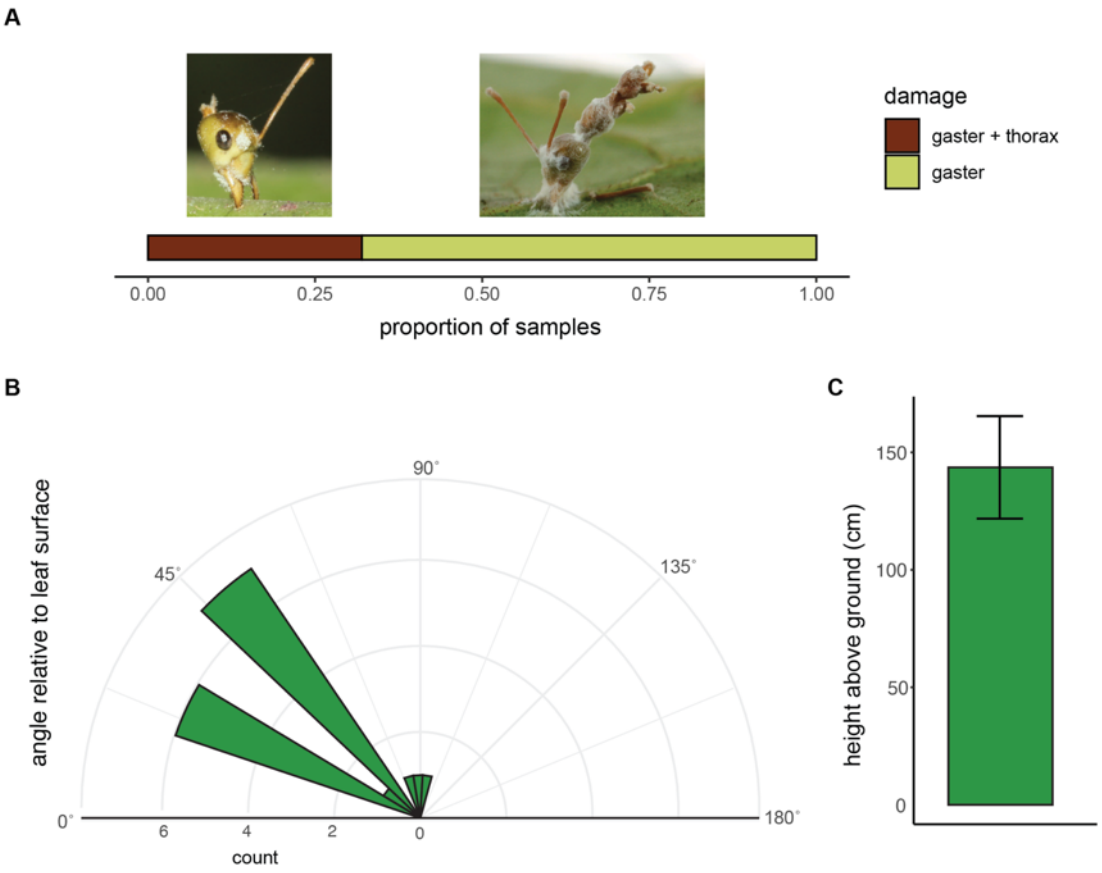


Figure 5-2. Characterizing the manipulation niche for *Ophiocordyceps oecophyllae.*

A) Proportion of zombie ant cadavers (n=39) found that were missing a gaster or missing a gaster and thorax. **B)** Angle of zombie ant cadaver's body relative to leaf surface (n=17) **C)** Average height above ground zombie ant cadavers were found attached to leaves.

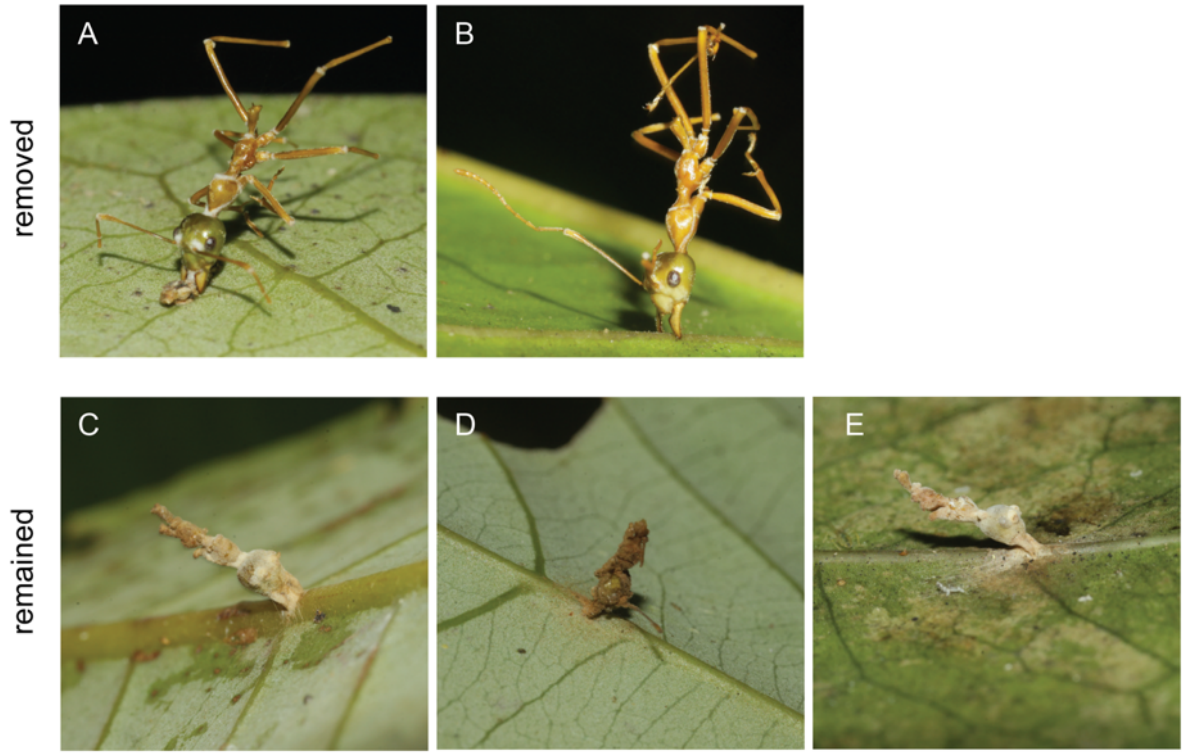


Figure 5-3. Images of zombie green tree ants.

A-B) *Oecophylla smaragdina* ants infected with *Ophiocordyceps oecophyllae* with a fresh-looking appearance that were removed during study period and **C-E)** infected ants with an older looking appearance that were not removed during the study period.

Chapter 6

Discussion

Summary

The *Ophiocordyceps* fungi are manipulative fungal parasites of ants. Infected ants are known as zombie ants and are manipulated to leave the nest to die. Manipulation is likely necessary to infect ants because of behavioral defenses limiting transmission within the nest. Throughout this dissertation, I emphasize how transmission outside of the nest helps parasites avoid colony defenses.

I began by investigating the dynamics outside of the nest in chapter 2 by studying how ants are foraging in an area of high disease pressure. On foraging trails, I found that most ants have similar trajectories and walk over the same areas as other ants, but there is a subset of foragers that tends to explore new areas. I suggest the explorative ants have a higher risk of exposure to parasites, while having the majority of ants walk in a similar manner could lessen the probability of transmission for most ants. Chapter 3 studied this system further and demonstrated that infected ants are likely turning more than most foragers. A higher frequency in turning for zombie ants could be adaptive as it helps the infectious host stay close to the nest and foraging trails, allowing spores to be released in area where they are more likely to be encountered by future ants. Interestingly, the group of ants discovered in chapter 2 with high turning rates (curvy ants – Fig. 2-2B) is similar to the expected zombie ant movement predicted by chapter 3 (overlap in Fig. 3-2A).

Chapter 4 explored a different system of the ant *Cephalotes atratus* infected with the parasite *Ophiocordyceps kniphofioides.* Infected ants are found biting at the base of trees, and I

found they die on specific trees and highly aggregated on the tree surfaces. Infected ants show some evidence of being attracted to the infected cadavers and this could lead to the aggregated distribution. Some cadavers were also removed by conspecifics, suggesting that social immunity behaviors extend outside of the nest.

Chapter 5 additionally demonstrates the potential pathogen clean up ability of ants outside of the nest, as 100% of green tree ants (*Oecophylla smaragdina*) infected with *Ophiocordyceps oecophyllae* were found damaged. Video recordings captured green tree ants removing infected cadavers from the leaves, eliminating the infectious material in the area. Interestingly, these cadavers were exceedingly rare, especially considering the abundance of the host ant and the density of cadavers found in the other two systems explored in this dissertation.

This last system shows how even after evolving highly specialized manipulation, a parasite can still face barriers when infecting ants. Certain strategies seem to work better than others, demonstrated by the large number of *O. kniphofioides* cadavers found in chapter 4. The multiple spore types produced by *O. kniphofioides*, particularly the type that can remain on the substrate surface after cadaver removal, might improve the success of the parasite. Moreover, the distance manipulated away from the nest might be important, such as how *O. kniphofioides* infected cadavers are found at the base of a tree while the host ant is mostly in the canopy. Location is also adaptive for the system discussed in chapters 2-3, *Ophiocordyceps camponoti-rufipedis*, as it allows the fungus to distribute spores onto the foraging trails that ants leaving the nest walk upon. Green tree ants also make trails, but *O. oecophyllae* does not produce a fruiting body and stalk like *Ophiocordyceps camponoti-rufipedis*, so it likely needs a host to touch the cadaver for transmission. Thus, the different biting locations and spore types produced could explain the relative success of the zombie ant fungi species studied.

Miscellaneous observations

In chapter 2, a variety of times I observed small arthropods, such as a spider (Supp. Video S5-1) and a cockroach (Supp. Video S5-2), following ants on the trails. Interestingly, the spider followed the ant's trajectories quite closely and even appeared to touch the ants without negative consequences. Other times, if an insect walked on the trunk trails, I observed the ants aggressively chasing it away. It would be interesting to further investigate the myrmecophile trail behavior. Possible directions include looking into the nature of their relationship (commensal/symbiotic/predatory), the mathematical similarity of their movements, how they avoid ant aggression, and what senses they primarily use to follow the ants.

When taking photographs of zombie ants, I often discovered small invertebrates next to the cadavers (Fig. 6-1). These small creatures could simply happen to be in the area at the time, but many were located directly on or below the cadaver. Perhaps these invertebrates could have an influence on the fungus growing from the cadaver. The invertebrates may eat the spores of the fungus or could even vector the spores to a new location, as mites are known to vector spores. A particularly interesting possibility is that the invertebrates could be introducing other fungal species, as zombie ant fungi are often parasitized by fungal 'hyperparasites' (example in Fig. 1-1C). Additionally, ant cadavers often have holes in the gaster or other parts of the body, so the ant itself may serve as a nutritional source. Future work could investigate the relationship between cadavers and the small invertebrates that interact with them.

Figure 6-1. Example invertebrates observed near zombie ant cadavers.

Future directions

Conspecific removal behavior

Further investigation of the cadaver removal behavior observed in the turtle (chapter 4) and green (chapter 5) ant systems would be useful to understand when infected cadavers are removed and how this influences the transmission cycle. Does the amount of fungal growth impact the removal behavior? What happens to the cadaver once removed? Do the removing ants become infected?

Production of different spore types

The transmission cycle should also be investigated from the perspective of the spores produced by the cadavers. The species *Ophiocordyceps kniphofioides* studied in chapter 4 produces a variety of different spore types, some that appear adapted for aerial transmission, and others for touch or rain dispersal. It would be interesting to quantify the rates of transmission of

these different spore types and if the production of the different types can be induced by environmental conditions.

Evolution of extended phenotypes

There are trends within the zombie ant phylogeny, with most ants biting onto leaves in the *O. unilateralis* group and tree trunks in the *O. kniphofioides*, although there are exceptions in each group. Additionally, there are examples of ants not biting at all or biting underground or inside logs. Future work should look further into how these different extended phenotypes evolve. Work in this direction might additionally provide broad insights into the evolution of parasite manipulation.

Concluding remarks

Overall, more work is needed to understand the interaction between sociality and disease risk. This dissertation shows how a fungal parasite can successfully infect ants, but does infection have fitness consequences for the host? Typically, only foraging workers are infected, which is only a fraction of the colony's population. Successfully avoiding epidemics might be one of the reasons ants are abundant in many ecosystems. However, many studies on social insect disease dynamics use abnormally high pathogen loads and focus on transmission within the nest, which may miss part of the puzzle. Studies mimicking natural conditions and using host-parasite pairs found in nature are crucial to new insights related to infectious disease in social insects. Moreover, with climate change and anthropogenic activities threatening many of the earth's biodiverse areas, it is imperative we understand and appreciate the diversity in social insect parasites.

Supplementary videos for chapter 6**:** https://doi.org/10.26207/7jm4-1z59

References

Adler, F. R., & Gordon, D. M. (1992). Information Collection and Spread by Networks of Patrolling Ants. *The American Naturalist*, *140*(3), 373–400. https://doi.org/10.1086/285418

Adler, L. S., Michaud, K. M., Ellner, S. P., McArt, S. H., Stevenson, P. C., & Irwin, R. E. (2018). Disease where you dine: Plant species and floral traits associated with pathogen transmission in bumble bees. *Ecology*, *99*(11), 2535–2545. https://doi.org/10.1002/ecy.2503

Alexander, R. D. (1974). The evolution of social behavior. *Annual Review of Ecology and Systematics*, *5*(1), 325–383.

Alger, S. A., Burnham, P. A., & Brody, A. K. (2019). Flowers as viral hot spots: Honey bees (Apis mellifera) unevenly deposit viruses across plant species. *PLOS ONE*, *14*(9), e0221800. https://doi.org/10.1371/journal.pone.0221800

Almeida, P. J. A. L., Vieira, M. V., Kajin, M., Forero-Medina, G., & Cerqueira, R. (2010). Indices of movement behaviour: Conceptual background, effects of scale and location errors. *Zoologia*, *27*(5). http://submission.scielo.br/index.php/zool/article/view/23239

Ame, J.-M., Rivault, C., & Deneubourg, J.-L. (2004). Cockroach aggregation based on strain odour recognition. *Animal Behaviour*, *68*(4), 793–801. https://doi.org/10.1016/j.anbehav.2004.01.009

Andersen, S. B., Gerritsma, S., Yusah, K. M., Mayntz, D., Hywel-Jones, N. L., Billen, J., Boomsma, J. J., & Hughes, D. P. (2009). The Life of a Dead Ant: The Expression of an

Adaptive Extended Phenotype. *The American Naturalist*, *174*(3), 424–433. https://doi.org/10.1086/603640

Anderson, C., & Jadin, J. L. V. (2001). The adaptive benefit of leaf transfer in Atta colombica. *Insectes Sociaux*, *48*(4), 404–405. https://doi.org/10.1007/PL00001798

Anderson, R. M., & May, R. M. (1979). Population biology of infectious diseases: Part I. *Nature*, *280*(5721), 361–367. https://doi.org/10.1038/280361a0

Araújo, J., & Hughes, D. (2017). *The fungal spore: Myrmecophilous Ophiocordyceps as a case study*. CRC Press, USA.

Araújo, J. P. M., Evans, H. C., Kepler, R., & Hughes, D. P. (2018). Zombie-ant fungi across continents: 15 new species and new combinations within Ophiocordyceps. I. Myrmecophilous hirsutelloid species. *Studies in Mycology*, *90*, 119–160. https://doi.org/10.1016/j.simyco.2017.12.002

Araújo, J. P. M., & Hughes, D. (2017). The fungal spore: Myrmecophilous Ophiocordyceps as a case study. In J. Dighton & J. F. White (Eds.), *The Fungal Community: Its Organization and Role in the Ecosystem, Fourth Edition* (pp. 359–367). CRC Press.

Araújo, J. P. M., & Hughes, D. P. (2019). Zombie-Ant Fungi Emerged from Non-manipulating, Beetle-Infecting Ancestors. *Current Biology*, *29*(21), 3735-3738.e2. https://doi.org/10.1016/j.cub.2019.09.004

Baldauf, S. A., Thünken, T., Frommen, J. G., Bakker, T. C. M., Heupel, O., & Kullmann, H. (2007). Infection with an acanthocephalan manipulates an amphipod's reaction to a fish predator's odours. *International Journal for Parasitology*, *37*(1), 61–65. https://doi.org/10.1016/j.ijpara.2006.09.003

Baracchi, D., Fadda, A., & Turillazzi, S. (2012). Evidence for antiseptic behaviour towards sick adult bees in honey bee colonies. *Journal of Insect Physiology*, *58*(12), 1589–1596. https://doi.org/10.1016/j.jinsphys.2012.09.014

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Benhamou, S. (2004). How to reliably estimate the tortuosity of an animal's path: Straightness, sinuosity, or fractal dimension? *Journal of Theoretical Biology*, *229*(2), 209–220. https://doi.org/10.1016/j.jtbi.2004.03.016

Berdoy, M., Webster, J. P., & Macdonald, D. W. (2000). Fatal attraction in rats infected with Toxoplasma gondii. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1452), 1591–1594. https://doi.org/10.1098/rspb.2000.1182

Biesmeijer, J. C., & de Vries, H. (2001). Exploration and exploitation of food sources by social insect colonies: A revision of the scout-recruit concept. *Behavioral Ecology and Sociobiology*, *49*(2), 89–99. https://doi.org/10.1007/s002650000289

Boer, P. (2008). Observations of summit disease in Formica rufa Linnaeus, 1761 (Hymenoptera: Formicidae). *Myrmecological News*, *11*, 63–66.

Bolton, B., Alpert, G., Ward, P. S., & Naskrecki, P. (2006). Bolton's Catalogue of Ants of the World. *Cambridge: Harvard*.

Boomsma, J. J., Jensen, A. B., Meyling, N. V., & Eilenberg, J. (2014). Evolutionary interaction networks of insect pathogenic fungi. *Annual Review of Entomology*, *59*, 467–485.

Boomsma, J., Schmid-Hempel, P., & Hughes, W. (2005). Life histories and parasite pressure across the major groups of social insects. *Insect Evolutionary Ecology: Proceedings of the Royal Entomological Society's 22nd Symposium*, 139–175.

Brütsch, T., Jaffuel, G., Vallat, A., Turlings, T. C. J., & Chapuisat, M. (2017). Wood ants produce a potent antimicrobial agent by applying formic acid on tree-collected resin. *Ecology and Evolution*, *7*(7), 2249–2254. https://doi.org/10.1002/ece3.2834

Burand, J. P., Tan, W., Kim, W., Nojima, S., & Roelofs, W. (2005). Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female Helicoverpa zea moths. *Journal of Insect Science*, *5*(1). https://doi.org/10.1093/jis/5.1.6

Burd, M., & Aranwela, N. (2003). Head-on encounter rates and walking speed of foragers in leaf-cutting ant traffic. *Insectes Sociaux*, *50*(1), 3–8. https://doi.org/10.1007/s000400300001

Buschinger, A. (2009). Social parasitism among ants: A review (Hymenoptera: Formicidae). *Myrmecological News*, *12*(3), 219–235.

Bution, M. L., & Caetano, F. H. (2008). Ileum of the Cephalotes ants: A specialized structure to harbor symbionts microorganisms. *Micron*, *39*(7), 897–909. https://doi.org/10.1016/j.micron.2007.11.008

Carney, W. P. (1969). Behavioral and Morphological Changes in Carpenter Ants Harboring Dicrocoeliid Metacercariae. *The American Midland Naturalist*, *82*(2), 605–611. https://doi.org/10.2307/2423801

Cezilly, F., Gregoire, A., & Bertin, A. (2000). Conflict between co-occurring manipulative parasites? An experimental study of the joint influence of two acanthocephalan parasites on the behaviour of Gammarus pulex. *Parasitology*, *120*(6), 625–630. https://doi.org/10.1017/S0031182099005910

Chen, J., Alber, M. S., & Chen, D. Z. (2016). A Hybrid Approach for Segmentation and Tracking of Myxococcus Xanthus Swarms. *IEEE Transactions on Medical Imaging*, *35*(9), 2074–2084. https://doi.org/10.1109/TMI.2016.2548490

Chen, J., Harvey, C. W., Alber, M. S., & Chen, D. Z. (2014). A Matching Model Based on Earth Mover's Distance for Tracking Myxococcus Xanthus. *Medical Image Computing and Computer-Assisted Intervention – MICCAI 2014*, 113–120. https://doi.org/10.1007/978-3-319-10470-6_15

Choe, J. C., & Crespi, B. J. (1997). *The evolution of social behaviour in insects and arachnids*. Cambridge University Press.

Corn, M. L. (1976). *The Ecology and Behavior of Cephalotes Atratus, a Neotropical Ant (hymenoptera: Formicidae)*. [Ph.D.]. Harvard University.

Corn, M. L. (1980). Polymorphism and polyethism in the neotropical antCephalotes Atratus (L.). *Insectes Sociaux*, *27*(1), 29–42. https://doi.org/10.1007/BF02224519

Couzin, I. D., & Franks, N. R. (2003). Self-organized lane formation and optimized traffic flow in army ants. *Proceedings of the Royal Society B. Biological Sciences*.

Crawford, D. L., & Rissing, S. W. (1983). Regulation of recruitment by individual scouts inFormica oreas Wheeler (Hymenoptera, Formicidae). *Insectes Sociaux*, *30*(2), 177–183. https://doi.org/10.1007/BF02223867

Cremer, S., Armitage, S. A. O., & Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, *17*(16), R693–R702. https://doi.org/10.1016/j.cub.2007.06.008

Crist, T., & MacMahon, J. (1991). Individual foraging components of harvester ants: Movement patterns and seed patch fidelity. *Insectes Sociaux*, *38*(4), 379–396.

Crozier, R. H., Newey, P. S., Schluens, E. A., Robson, S. K., & others. (2010). A masterpiece of evolution–Oecophylla weaver ants (Hymenoptera: Formicidae). *Myrmecological News*, *13*(5).

Csata, E., Czekes, Z., Erōs, K., Német, E., Hughes, M., Csosz, S., & Marko, B. (2013). Comprehensive survey of Romanian myrmecoparasitic fungi: New species, biology and distribution. *North-Western Journal of Zoology*.

Cushing, P. E. (1997). Myrmecomorphy and Myrmecophily in Spiders: A Review. *The Florida Entomologist*, *80*(2), 165–193. JSTOR. https://doi.org/10.2307/3495552

d'Ettorre, P., Carere, C., Demora, L., Le Quinquis, P., Signorotti, L., & Bovet, D. (2017). Individual differences in exploratory activity relate to cognitive judgement bias in

carpenter ants. *Behavioural Processes*, *134*, 63–69. https://doi.org/10.1016/j.beproc.2016.09.008

Davidson, D. W., Cook, S. C., Snelling, R. R., & Chua, T. H. (2003). Explaining the Abundance of Ants in Lowland Tropical Rainforest Canopies. *Science*, *300*(5621), 969–972. https://doi.org/10.1126/science.1082074

De Andrade, M. L., & Urbani, C. B. (1999). Diversity and adaptation in the ant genus Cephalotes, past and present (Hymenoptera, Formicidae). *Stuttgarter Beiträge Zur Naturkunde.*, *271*, 1–889.

de Bekker, C., Ohm, R. A., Loreto, R. G., Sebastian, A., Albert, I., Merrow, M., Brachmann, A., & Hughes, D. P. (2015). Gene expression during zombie ant biting behavior reflects the complexity underlying fungal parasitic behavioral manipulation. *BMC Genomics*, *16*, 620. https://doi.org/10.1186/s12864-015-1812-x

Del-Claro, K., & Oliveira, P. S. (1999). Ant-Homoptera Interactions in a Neotropical Savanna: The Honeydew-Producing Treehopper, Guayaquila xiphias (Membracidae), and its Associated Ant Fauna on Didymopanax vinosum (Araliaceae) 1. *Biotropica*, *31*(1), 135–144.

Deneubourg, J. L., & Goss, S. (1989). Collective patterns and decision-making. *Ethology Ecology & Evolution*, *1*(4), 295–311. https://doi.org/10.1080/08927014.1989.9525500

Detrain, C. (1990). Field study on foraging by the polymorphic ant species, Pheidole pallidula. *Insectes Sociaux*, *37*(4), 315–332. https://doi.org/10.1007/BF02225995

Detrain, C., Tasse, O., Versaen, M., & Pasteels, J. M. (2000). A field assessment of optimal foraging in ants: Trail patterns and seed retrieval by the European harvester ant Messor barbarus. *Insectes Sociaux*, *47*(1), 56–62. https://doi.org/10.1007/s000400050009

Diez, L., Lejeune, P., & Detrain, C. (2014). Keep the nest clean: Survival advantages of corpse removal in ants. *Biology Letters*, *10*(7), 20140306. https://doi.org/10.1098/rsbl.2014.0306

Dreisig, H. (2000). Defense by exploitation in the Florida carpenter ant, Camponotus floridanus, at an extrafloral nectar resource. *Behavioral Ecology and Sociobiology*, *47*(4), 274–279.

Duffy, J. E. (2007). Ecology and evolution of eusociality in sponge-dwelling shrimp. In J. E. Duffy & M. Thiel (Eds.), *Evolutionary ecology of social and sexual systems: Crustaceans as model organisms* (pp. 387–409). Oxford University Press.

Dussutour, A., Fourcassié, V., Helbing, D., & Deneubourg, J.-L. (2004). Optimal traffic organization in ants under crowded conditions. *Nature*, *428*(6978), 70–73. https://doi.org/10.1038/nature02345

Edelstein-Keshet, L. (1994). Simple models for trail-following behaviour; Trunk trails versus individual foragers. *Journal of Mathematical Biology*, *32*(4), 303–328. https://doi.org/10.1007/BF00160163

Edelstein-Keshet, L., Watmough, J., & Ermentrout, G. B. (1995). Trail following in ants: Individual properties determine population behaviour. *Behavioral Ecology and Sociobiology*, *36*(2), 119–133. https://doi.org/10.1007/BF00170717

Erwin, T. L. (1989). Canopy arthropod biodiversity: A chronology of sampling techniques and results. *Revista Peruana de Entomologia*, *32*, 71–77.

Evans, H. C., Elliot, S. L., & Hughes, D. P. (2011). Hidden Diversity Behind the Zombie-Ant Fungus Ophiocordyceps unilateralis: Four New Species Described from Carpenter Ants in Minas Gerais, Brazil. *PLOS ONE*, *6*(3), e17024. https://doi.org/10.1371/journal.pone.0017024

Evans, H. C., & Samson, R. A. (1982). Cordyceps species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems I. The Cephalotes (Myrmicinae) complex. *Transactions of the British Mycological Society*, *79*(3), 431–453. https://doi.org/10.1016/S0007-1536(82)80037-5

Evans, H. C., & Samson, R. A. (1984). Cordyceps species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems II. The Camponotus (Formicinae) complex. *Transactions of the British Mycological Society*, *82*(1), 127–150. https://doi.org/10.1016/S0007-1536(84)80219-3

Evison, S. E. F., Hart, A. G., & Jackson, D. E. (2008). Minor workers have a major role in the maintenance of leafcutter ant pheromone trails. *Animal Behaviour*, *75*(3), 963–969. https://doi.org/10.1016/j.anbehav.2007.07.013

Feener, D. H., & Moss, K. A. G. (1990). Defense against parasites by hitchhikers in leaf-cutting ants: A quantitative assessment. *Behavioral Ecology and Sociobiology*, *26*(1), 17–29. https://doi.org/10.1007/BF00174021

Feldmeyer, B., Mazur, J., Beros, S., Lerp, H., Binder, H., & Foitzik, S. (2016). Gene expression patterns underlying parasite-induced alterations in host behaviour and life history. *Molecular Ecology*, *25*(2), 648–660. https://doi.org/10.1111/mec.13498

Fernández-Marín, H., Zimmerman, J. K., Rehner, S. A., & Wcislo, W. T. (2006). Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1594), 1689–1695. https://doi.org/10.1098/rspb.2006.3492

Figueroa, L. L., Blinder, M., Grincavitch, C., Jelinek, A., Mann, E. K., Merva, L. A., Metz, L. E., Zhao, A. Y., Irwin, R. E., McArt, S. H., & Adler, L. S. (2019). Bee pathogen transmission dynamics: Deposition, persistence and acquisition on flowers. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1903), 20190603. https://doi.org/10.1098/rspb.2019.0603

Fineberg, H. V. (2014). Pandemic Preparedness and Response—Lessons from the H1N1 Influenza of 2009. *New England Journal of Medicine*, *370*(14), 1335–1342. https://doi.org/10.1056/NEJMra1208802

Fittkau, E. J., & Klinge, H. (1973). On Biomass and Trophic Structure of the Central Amazonian Rain Forest Ecosystem. *Biotropica*, *5*(1), 2–14. https://doi.org/10.2307/2989676

Flanagan, T. P., Pinter-Wollman, N. M., Moses, M. E., & Gordon, D. M. (2013). Fast and Flexible: Argentine Ants Recruit from Nearby Trails. *PLOS ONE*, *8*(8), e70888. https://doi.org/10.1371/journal.pone.0070888

Fouks, B., & Lattorff, H. M. G. (2011). Recognition and Avoidance of Contaminated Flowers by Foraging Bumblebees (Bombus terrestris). *PLOS ONE*, *6*(10), e26328. https://doi.org/10.1371/journal.pone.0026328

Fourcassié, V., Dussutour, A., & Deneubourg, J.-L. (2010). Ant traffic rules. *Journal of Experimental Biology*, *213*(14), 2357–2363. https://doi.org/10.1242/jeb.031237

Frank, S. A. (1996). Models of Parasite Virulence. *The Quarterly Review of Biology*, *71*(1), 37–78. https://doi.org/10.1086/419267

Franks, N. R., & Deneubourg, J.-L. (1997). Self-organizing nest construction in ants: Individual worker behaviour and the nest's dynamics. *Animal Behaviour*, *54*(4), 779–796. https://doi.org/10.1006/anbe.1996.0496

Franks, N. R., Richardson, T. O., Keir, S., Inge, S. J., Bartumeus, F., & Sendova-Franks, A. B. (2010). Ant search strategies after interrupted tandem runs. *Journal of Experimental Biology*, *213*(10), 1697–1708. https://doi.org/10.1242/jeb.031880

Fredericksen, M. A., Zhang, Y., Hazen, M. L., Loreto, R. G., Mangold, C. A., Chen, D. Z., & Hughes, D. P. (2017). Three-dimensional visualization and a deep-learning model reveal complex fungal parasite networks in behaviorally manipulated ants. *Proceedings of the National Academy of Sciences*, *114*(47), 12590–12595. https://doi.org/10.1073/pnas.1711673114

Gal, R., & Libersat, F. (2008). A Parasitoid Wasp Manipulates the Drive for Walking of Its Cockroach Prey. *Current Biology*, *18*(12), 877–882. https://doi.org/10.1016/j.cub.2008.04.076

Gal, R., Rosenberg, L. A., & Libersat, F. (2005). Parasitoid wasp uses a venom cocktail injected into the brain to manipulate the behavior and metabolism of its cockroach prey. *Archives of Insect Biochemistry and Physiology*, *60*(4), 198–208. https://doi.org/10.1002/arch.20092

Gordon, D. M. (1991). Behavioral Flexibility and the Foraging Ecology of Seed-Eating Ants. *The American Naturalist*, *138*(2), 379–411. https://doi.org/10.1086/285223

Gordon, D. M. (1995). The expandable network of ant exploration. *Animal Behaviour*, *50*(4), 995–1007. https://doi.org/10.1016/0003-3472(95)80100-6

Gordon, D. M. (2013). The rewards of restraint in the collective regulation of foraging by harvester ant colonies. *Nature*, *498*(7452), 91–95.

Gordon, D. M., Guetz, A., Greene, M. J., & Holmes, S. (2011). Colony variation in the collective regulation of foraging by harvester ants. *Behavioral Ecology*, *22*(2), 429–435. https://doi.org/10.1093/beheco/arq218

Goss, S., Aron, S., Deneubourg, J. L., & Pasteels, J. M. (1989). Self-organized shortcuts in the Argentine ant. *Naturwissenschaften*, *76*(12), 579–581. https://doi.org/10.1007/BF00462870

Goulson, D. (1997). Wipfelkrankheit: Modification of host behaviour during baculoviral infection. *Oecologia*, *109*(2), 219–228. https://doi.org/10.1007/s004420050076

Gracia, E. S., Bekker, C. de, Hanks, E. M., & Hughes, D. P. (2018). Within the fortress: A specialized parasite is not discriminated against in a social insect society. *PLOS ONE*, *13*(2), e0193536. https://doi.org/10.1371/journal.pone.0193536

Graystock, P., Goulson, D., & Hughes, W. O. H. (2015). Parasites in bloom: Flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proc. R. Soc. B*, *282*(1813), 20151371. https://doi.org/10.1098/rspb.2015.1371

Graystock, P., & Hughes, W. O. H. (2011). Disease resistance in a weaver ant, Polyrhachis dives, and the role of antibiotic-producing glands. *Behavioral Ecology and Sociobiology*, *65*(12), 2319–2327. https://doi.org/10.1007/s00265-011-1242-y

Grimm, V., Berger, U., Bastiansen, F., Eliassen, S., Ginot, V., Giske, J., Goss-Custard, J., Grand, T., Heinz, S. K., Huse, G., Huth, A., Jepsen, J. U., Jørgensen, C., Mooij, W. M., Müller, B., Pe'er, G., Piou, C., Railsback, S. F., Robbins, A. M., … DeAngelis, D. L. (2006). A standard protocol for describing individual-based and agent-based models. *Ecological Modelling*, *198*(1), 115–126. https://doi.org/10.1016/j.ecolmodel.2006.04.023

Grimm, V., Berger, U., DeAngelis, D. L., Polhill, J. G., Giske, J., & Railsback, S. F. (2010). The ODD protocol: A review and first update. *Ecological Modelling*, *221*(23), 2760–2768. https://doi.org/10.1016/j.ecolmodel.2010.08.019

Hamilton, W. (1964). *The Genetical Evolution of Social Behaviour, Pts. I and II. Journal of Theoretical Biology 7: 1-52*.

Hart, A., & Jackson, D. E. (2006). U-turns on ant pheromone trails. *Current Biology*, *16*(2), R42–R43. https://doi.org/10.1016/j.cub.2006.01.015

Hays, J. N. (2005). *Epidemics and pandemics: Their impacts on human history*. Abc-clio.

He, K., Gkioxari, G., Dollár, P., & Girshick, R. (2017). Mask R-CNN. *2017 IEEE International Conference on Computer Vision (ICCV)*, 2980–2988. https://doi.org/10.1109/ICCV.2017.322

Heller, N. E., & Gordon, D. M. (2006). Seasonal spatial dynamics and causes of nest movement in colonies of the invasive Argentine ant (Linepithema humile). *Ecological Entomology*. http://agris.fao.org/agris-search/search.do?recordID=US201301101206

Hesketh, H., Roy, H., Eilenberg, J., Pell, J., & Hails, R. (2010). Challenges in modelling complexity of fungal entomopathogens in semi-natural populations of insects. *BioControl*, *55*(1), 55–73.

Heussler, V., Kaufmann, H., Glaser, I., Ducommun, D., Müller, C., & Dobbelaere, D. (1998). A DNA probe for the detection of Dicrocoelium dendriticum in ants of Formica spp. And Lasius spp. *Parasitology Research*, *84*(6), 505–508. https://doi.org/10.1007/s004360050437

Hölldobler, B., & Wilson, E. O. (1990). *The ants.* Springer Verlag; CABDirect.

Hölldobler, B. (1981). Foraging and spatiotemporal territories in the honey ant Myrmecocystus mimicuswheeler (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*, *9*(4), 301–314. https://doi.org/10.1007/BF00299887

Hölldobler, B. (1983). Territorial Behavior in the Green Tree Ant (Oecophylla smaragdina). *Biotropica*, *15*(4), 241–250. JSTOR. https://doi.org/10.2307/2387648

Holway, D. A., & Case, T. J. (2000). Mechanisms of dispersed central-place foraging in polydomous colonies of the Argentine ant. *Animal Behaviour*, *59*(2), 433–441. https://doi.org/10.1006/anbe.1999.1329

Hoover, K., Grove, M., Gardner, M., Hughes, D. P., McNeil, J., & Slavicek, J. (2011). A Gene for an Extended Phenotype. *Science*, *333*(6048), 1401–1401. https://doi.org/10.1126/science.1209199

Howard, J. J., Henneman, L. M., Cronin, G., Fox, J. A., & Hormiga, G. (1996). Conditioning of scouts and recruits during foraging by a leaf-cutting ant,Atta colombica. *Animal Behaviour*, *52*(2), 299–306. https://doi.org/10.1006/anbe.1996.0175

Howard, J. J. (2001). Costs of trail construction and maintenance in the leaf-cutting ant Atta columbica. *Behavioral Ecology and Sociobiology*, *49*(5), 348–356. https://doi.org/10.1007/s002650000314

Hu, Y., Sanders, J. G., Łukasik, P., D'Amelio, C. L., Millar, J. S., Vann, D. R., Lan, Y., Newton, J. A., Schotanus, M., Kronauer, D. J. C., Pierce, N. E., Moreau, C. S., Wertz, J. T., Engel, P., & Russell, J. A. (2018). Herbivorous turtle ants obtain essential nutrients from a conserved nitrogen-recycling gut microbiome. *Nature Communications*, *9*(1), 1–14. https://doi.org/10.1038/s41467-018-03357-y

Hughes, D. P. (2012). Parasites and the superorganism. In J. Brodeur, F. Thomas, & D. P. Hughes (Eds.), *Host Manipulation by Parasites* (pp. 140–154). Oxford University Press.

Hughes, D. P., Andersen, S. B., Hywel-Jones, N. L., Himaman, W., Billen, J., & Boomsma, J. J. (2011). Behavioral mechanisms and morphological symptoms of zombie ants dying from fungal infection. *BMC Ecology*, *11*, 13. https://doi.org/10.1186/1472-6785-11-13

Hughes, D. P., & Libersat, F. (2019). Parasite manipulation of host behavior. *Current Biology*, *29*(2), R45–R47. https://doi.org/10.1016/j.cub.2018.12.001

Hughes, D. P., Pierce, N. E., & Boomsma, J. J. (2008). Social insect symbionts: Evolution in homeostatic fortresses. *Trends in Ecology & Evolution*, *23*(12), 672–677. https://doi.org/10.1016/j.tree.2008.07.011

Humber, R. A. (2012). Identification of entomopathogenic fungi. *Manual of Techniques in Invertebrate Pathology*, *2*, 151–187.

Imirzian, N., Zhang, Y., Kurze, C., Loreto, R. G., Chen, D. Z., & Hughes, D. P. (2018). Computer vision and deep learning automates nocturnal rainforest ant tracking to provide insight into behavior and disease risk. *BioRxiv*, 454207. https://doi.org/10.1101/454207

Imirzian, N., Zhang, Y., Kurze, C., Loreto, R. G., Chen, D. Z., & Hughes, D. P. (2019). Automated tracking and analysis of ant trajectories shows variation in forager exploration. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-49655-3

Jackson, D. E., Holcombe, M., & Ratnieks, F. L. W. (2004). Trail geometry gives polarity to ant foraging networks. *Nature*, *432*(7019), 907. https://doi.org/10.1038/nature03105

Jaffe, K., & Sanchez, C. (1984). On the nestmate-recognition system and territorial marking behaviour in the antCamponotus rufipes. *Insectes Sociaux*, *31*(3), 302–315. https://doi.org/10.1007/BF02223614

Jarvis, J. U. M., & Bennett, N. C. (1993). Eusociality has evolved independently in two genera of bathyergid mole-rats—But occurs in no other subterranean mammal. *Behavioral Ecology and Sociobiology*, *33*(4), 253–260. https://doi.org/10.1007/BF02027122

John, A., Schadschneider, A., Chowdhury, D., & Nishinari, K. (2009). Trafficlike Collective Movement of Ants on Trails: Absence of a Jammed Phase. *Physical Review Letters*, *102*(10), 108001. https://doi.org/10.1103/PhysRevLett.102.108001

Kaner, J., & Schaack, S. (2016). Understanding Ebola: The 2014 epidemic. *Globalization and Health*, *12*(1), 53. https://doi.org/10.1186/s12992-016-0194-4

Keesey, I. W., Koerte, S., Khallaf, M. A., Retzke, T., Guillou, A., Grosse-Wilde, E., Buchon, N., Knaden, M., & Hansson, B. S. (2017). Pathogenic bacteria enhance dispersal through alteration of Drosophila social communication. *Nature Communications*, *8*. https://doi.org/10.1038/s41467-017-00334-9

Kobmoo, N., Mongkolsamrit, S., Tasanathai, K., Thanakitpipattana, D., & Luangsa-Ard, J. (2012). Molecular phylogenies reveal host-specific divergence of Ophiocordyceps unilateralis sensu lato following its host ants. *Molecular Ecology*, *21*(12), 3022–3031.

Kohler, M., & Wehner, R. (2005). Idiosyncratic route-based memories in desert ants, Melophorus bagoti: How do they interact with path-integration vectors? *Neurobiology of Learning and Memory*, *83*(1), 1–12. https://doi.org/10.1016/j.nlm.2004.05.011

Konrad, M., Pull, C. D., Metzler, S., Seif, K., Naderlinger, E., Grasse, A. V., & Cremer, S. (2018). Ants avoid superinfections by performing risk-adjusted sanitary care. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(11), 2782–2787. https://doi.org/10.1073/pnas.1713501115

Kost, C., Oliveira, E. G. de, Knoch, T. A., & Wirth, R. (2005). Spatio-temporal permanence and plasticity of foraging trails in young and mature leaf-cutting ant colonies (Atta spp.). *Journal of Tropical Ecology*, *21*(6), 677–688. https://doi.org/10.1017/S0266467405002592

Krull, W. H., & Mapes, C. R. (1953). Studies on the biology of Dicrocoelium dendritiaum (Rudolphi, 1819) Looss, 1899 (Trernatoda: Dicrocoeliidae), including its relation to the intermediate host, Cionella lubrica (Müller). IX. Notes on the cyst, metacercaria, and infection in the ant, Formica fusca. *Cornell Veterinarian*, *43*(3), 389–410.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, *82*(13). https://doi.org/10.18637/jss.v082.i13

Latty, T., & Beekman, M. (2013). Keeping track of changes: The performance of ant colonies in dynamic environments. *Animal Behaviour*, *85*(3), 637–643. https://doi.org/10.1016/j.anbehav.2012.12.027

Libersat, F., Delago, A., & Gal, R. (2009). Manipulation of host behavior by parasitic insects and insect parasites. *Annual Review of Entomology*, *54*, 189–207.

Lighton, J. R. B., & Duncan, F. D. (2002). Energy Cost of Locomotion: Validation of Laboratory Data by in Situ Respirometry. *Ecology*, *83*(12), 3517–3522. https://doi.org/10.1890/0012-9658(2002)083[3517:ECOLVO]2.0.CO;2

Loreto, R. G., Araújo, J. P. M., Kepler, R. M., Fleming, K. R., Moreau, C. S., & Hughes, D. P. (2018). Evidence for convergent evolution of host parasitic manipulation in response to environmental conditions. *Evolution*, *72*(10), 2144–2155. https://doi.org/10.1111/evo.13489

Loreto, R. G., Elliot, S. L., Freitas, M. L. R., Pereira, T. M., & Hughes, D. P. (2014). Long-Term Disease Dynamics for a Specialized Parasite of Ant Societies: A Field Study. *PLOS ONE*, *9*(8), e103516. https://doi.org/10.1371/journal.pone.0103516

Loreto, R. G., Hart, A. G., Pereira, T. M., Freitas, M. L. R., Hughes, D. P., & Elliot, S. L. (2013). Foraging ants trade off further for faster: Use of natural bridges and trunk trail permanency in carpenter ants. *Naturwissenschaften*, *100*(10), 957–963. https://doi.org/10.1007/s00114-013-1096-4

Lynch, J. F., Balinsky, E. C., & Vail, S. G. (1980). Foraging patterns in three sympatric forest ant species, Prenolepis imparis, Paratrechina melanderi and Aphaenogaster rudis (Hymenoptera: Formicidae). *Ecological Entomology*, *5*(4), 353–371. https://doi.org/10.1111/j.1365-2311.1980.tb01160.x

Małagocka, J., Jensen, A. B., & Eilenberg, J. (2017). Pandora formicae, a specialist ant pathogenic fungus: New insights into biology and taxonomy. *Journal of Invertebrate Pathology*, *143*, 108–114. https://doi.org/10.1016/j.jip.2016.12.007

Manga-González, M. Y., González-Lanza, C., Cabanas, E., & Campo, R. (2001). Contributions to and review of dicrocoeliosis, with special reference to the intermediate hosts of Dicrocoelium dendriticum. *Parasitology*, *123*(7), 91–114. https://doi.org/10.1017/S0031182001008204

Mangold, C. A., Ishler, M. J., Loreto, R. G., Hazen, M. L., & Hughes, D. P. (2019). Zombie ant death grip due to hypercontracted mandibular muscles. *Journal of Experimental Biology*, *222*(14), jeb200683. https://doi.org/10.1242/jeb.200683

Marikovsky, P. (1962). On some features of behavior of the ants Formica rufa L. infected with fungous disease. *Insectes Sociaux*, *9*(2), 173–179.

May, R. M., & Anderson, R. M. (1983). Epidemiology and genetics in the coevolution of parasites and hosts. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *219*(1216), 281–313. https://doi.org/10.1098/rspb.1983.0075

Meunier, J. (2015). Social immunity and the evolution of group living in insects. *Phil. Trans. R. Soc. B*, *370*(1669), 20140102. https://doi.org/10.1098/rstb.2014.0102

Michener, C. D. (1969). Comparative Social Behavior of Bees. *Annual Review of Entomology*, *14*(1), 299–342. https://doi.org/10.1146/annurev.en.14.010169.001503

Moffett, M. W. (1987). Ants that go with the flow: A new method of orientation by mass communication. *Naturwissenschaften*, *74*(11), 551–553. https://doi.org/10.1007/BF00367078

Moore, J. (2002). *Parasites and the Behavior of Animals*. Oxford University Press.

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L., & Schultz, T. R. (2005). The Evolution of Agriculture in Insects. *Annual Review of Ecology, Evolution, and Systematics*, *36*(1), 563–595. https://doi.org/10.1146/annurev.ecolsys.36.102003.152626

Nijhout, H. F. (1994). Insect Hormones Princeton Univ. *Press, Princeton, NJ*.

Okuno, M., Tsuji, K., Sato, H., & Fujisaki, K. (2012). Plasticity of grooming behavior against entomopathogenic fungus Metarhizium anisopliae in the ant Lasius japonicus. *Journal of Ethology*, *30*(1), 23–27. https://doi.org/10.1007/s10164-011-0285-x

Pearce-Duvet, J. M. C., Elemans, C. P. H., & Feener, D. H. (2011). Walking the line: Search behavior and foraging success in ant species. *Behavioral Ecology*, *22*(3), 501–509. https://doi.org/10.1093/beheco/arr001

Peng, R. K., Christian, K., & Gibb, K. (1995). The effect of the green ant, Oecophylla smaragdina (Hymenoptera: Formicidae), on insect pests of cashew trees in Australia. *Bulletin of Entomological Research*, *85*(2), 279–284. https://doi.org/10.1017/S0007485300034374

Peng, R. K., Christian, K., & Gibb, K. (1997). Distribution of the green ant, Oecophylla smaragdina (F.) (Hymenoptera: Formicidae), in relation to native vegetation and the insect pests in cashew plantations in Australia. *International Journal of Pest Management*, *43*(3), 203–211. https://doi.org/10.1080/096708797228690

Pereira, H., & Detrain, C. (2020). Pathogen avoidance and prey discrimination in ants. *Royal Society Open Science*, *7*(2), 191705. https://doi.org/10.1098/rsos.191705

Perlman, S. (2020). Another Decade, Another Coronavirus. *New England Journal of Medicine*, *382*(8), 760–762. https://doi.org/10.1056/NEJMe2001126

Pie, M. R., Rosengaus, R. B., & Traniello, J. F. A. (2004). Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. *Journal of Theoretical Biology*, *226*(1), 45–51. https://doi.org/10.1016/j.jtbi.2003.08.002

Ponton, F., Otálora-Luna, F., Lefèvre, T., Guerin, P. M., Lebarbenchon, C., Duneau, D., Biron, D. G., & Thomas, F. (2011). Water-seeking behavior in worm-infected crickets and reversibility of parasitic manipulation. *Behavioral Ecology*, *22*(2), 392–400. https://doi.org/10.1093/beheco/arq215

Pontoppidan, M.-B., Himaman, W., Hywel-Jones, N. L., Boomsma, J. J., & Hughes, D. P. (2009). Graveyards on the Move: The Spatio-Temporal Distribution of Dead Ophiocordyceps-Infected Ants. *PLOS ONE*, *4*(3), e4835. https://doi.org/10.1371/journal.pone.0004835

Porter, S. D., & Jorgensen, C. D. (1981). Foragers of the harvester ant, Pogonomyrmex owyheei: A disposable caste? *Behavioral Ecology and Sociobiology*, *9*(4), 247–256. https://doi.org/10.1007/BF00299879

Poulin, R. (1993). The disparity between observed and uniform distributions: A new look at parasite aggregation. *International Journal for Parasitology*, *23*(7), 937–944. https://doi.org/10.1016/0020-7519(93)90060-C

Poulin, R. (1995). “Adaptive” changes in the behaviour of parasitized animals: A critical review. *International Journal for Parasitology*, *25*(12), 1371–1383. https://doi.org/10.1016/0020-7519(95)00100-X

Poulin, R. (2010). Chapter 5 - Parasite Manipulation of Host Behavior: An Update and Frequently Asked Questions. In T. J. R. H. Jane Brockmann Marc Naguib, Katherine E. Wynne-Edwards, John C. Mitani and Leigh W. Simmons (Ed.), *Advances in the Study of Behavior* (Vol. 41, pp. 151–186). Academic Press.

Purkiss, T., & Lach, L. (2019). Pathogen spillover from Apis mellifera to a stingless bee. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1908), 20191071. https://doi.org/10.1098/rspb.2019.1071

Quevillon, L. E., & Hughes, D. P. (2018). Pathogens, parasites, and parasitoids of ants: A synthesis of parasite biodiversity and epidemiological traits. *BioRxiv*, 384495. https://doi.org/10.1101/384495

R Core Team. (2018). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Ravary, F., Lecoutey, E., Kaminski, G., Châline, N., & Jaisson, P. (2007). Individual Experience Alone Can Generate Lasting Division of Labor in Ants. *Current Biology*, *17*(15), 1308–1312. https://doi.org/10.1016/j.cub.2007.06.047

Reber, A., Purcell, J., Buechel, S. D., Buri, P., & Chapuisat, M. (2011). The expression and impact of antifungal grooming in ants. *Journal of Evolutionary Biology*, *24*(5), 954–964. https://doi.org/10.1111/j.1420-9101.2011.02230.x

Reid, C. R., Latty, T., & Beekman, M. (2012). Making a trail: Informed Argentine ants lead colony to the best food by U-turning coupled with enhanced pheromone laying. *Animal Behaviour*, *84*(6), 1579–1587.

Renucci, M., Tirard, A., & Provost, E. (2011). Complex undertaking behavior in Temnothorax lichtensteini ant colonies: From corpse-burying behavior to necrophoric behavior. *Insectes Sociaux*, *58*(1), 9–16. https://doi.org/10.1007/s00040-010-0109-y

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E., & Pell, J. K. (2006). BIZARRE INTERACTIONS AND ENDGAMES: Entomopathogenic Fungi and Their Arthropod Hosts. *Annual Review of Entomology*, *51*(1), 331–357. https://doi.org/10.1146/annurev.ento.51.110104.150941

RStudio Team. (2016). *RStudio: Integrated Development Environment for R*. RStudio, Inc. http://www.rstudio.com/

Russell, J. A., Moreau, C. S., Goldman-Huertas, B., Fujiwara, M., Lohman, D. J., & Pierce, N. E. (2009). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences*, *106*(50), 21236–21241. https://doi.org/10.1073/pnas.0907926106

Sakolrak, B., Blatrix, R., Sangwanit, U., Arnamnart, N., Noisripoom, W., Thanakitpipattana, D., Buatois, B., Hossaert-McKey, M., & Kobmoo, N. (2018). Ant-produced chemicals are not responsible for the specificity of their Ophiocordyceps fungal pathogens. *Fungal Ecology*, *32*, 80–86. https://doi.org/10.1016/j.funeco.2017.11.005

Sakolrak, B., Blatrix, R., Sangwanit, U., & Kobmoo, N. (2018). Experimental infection of the ant Polyrhachis furcata with Ophiocordyceps reveals specificity of behavioural manipulation. *Fungal Ecology*, *33*, 122–124. https://doi.org/10.1016/j.funeco.2018.03.001

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Seeley, T. D. (1983). Division of labor between scouts and recruits in honeybee foraging. *Behavioral Ecology and Sociobiology*, *12*(3), 253–259. https://doi.org/10.1007/BF00290778

Seeley, T. D. (2009). *The wisdom of the hive: The social physiology of honey bee colonies*. Harvard University Press.

Shaw, D. J., & Dobson, A. P. (1995). Patterns of macroparasite abundance and aggregation in wildlife populations: A quantitative review. *Parasitology*, *111*(S1), S111–S133. https://doi.org/10.1017/S0031182000075855

Stroeymeyt, N., Casillas-Pérez, B., & Cremer, S. (2014). Organisational immunity in social insects. *Current Opinion in Insect Science*, *5*, 1–15. https://doi.org/10.1016/j.cois.2014.09.001

Stroeymeyt, N., Grasse, A. V., Crespi, A., Mersch, D. P., Cremer, S., & Keller, L. (2018). Social network plasticity decreases disease transmission in a eusocial insect. *Science*, *362*(6417), 941–945. https://doi.org/10.1126/science.aat4793

Sun, Q., & Zhou, X. (2013). Corpse Management in Social Insects. *International Journal of Biological Sciences*, *9*(3), 313–321. https://doi.org/10.7150/ijbs.5781

Theraulaz, G., Bonabeau, E., & Deneubourg, J.-L. (1998). The origin of nest complexity in social insects. *Complexity*, *3*(6), 15–25.

Thomas, F., Schmidt-Rhaesa, A., Martin, G., Manu, C., Durand, P., & Renaud, F. (2002). Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? *Journal of Evolutionary Biology*, *15*(3), 356–361. https://doi.org/10.1046/j.1420-9101.2002.00410.x

Thomas, J. A., & Settele, J. (2004). Butterfly mimics of ants. *Nature*, *432*(7015), 283–284. https://doi.org/10.1038/432283a

Torres-Contreras, H., & Canals, M. (2010). Effect of Colony, Patch Distance, And Trajectory Sense on Movement Complexity in Foraging Ants. *Journal of Insect Behavior*, *23*(4), 319–328. https://doi.org/10.1007/s10905-010-9216-x

Tragust, S., Mitteregger, B., Barone, V., Konrad, M., Ugelvig, L. V., & Cremer, S. (2013). Ants Disinfect Fungus-Exposed Brood by Oral Uptake and Spread of Their Poison. *Current Biology*, *23*(1), 76–82. https://doi.org/10.1016/j.cub.2012.11.034

Turchin, P. (1998). *Quantitative analysis of movement: Measuring and modeling population redistribution in animals and plants*. Sinauer Associates.

van Houte, S., Ros, V. I. D., & van Oers, M. M. (2013). Walking with insects: Molecular mechanisms behind parasitic manipulation of host behaviour. *Molecular Ecology*, *22*(13), 3458–3475. https://doi.org/10.1111/mec.12307

Villani, M. G., Allee, L. L., Preston-Wilsey, L., Consolie, N., Xia, Y., & Brandenburg, R. L. (2002). Use of Radiography and Tunnel Castings for Observing Mole Cricket (Orthoptera: Gryllotalpidae) Behavior in Soil. *American Entomologist*, *48*(1), 42–50. https://doi.org/10.1093/ae/48.1.42

Von Frisch, K. (1967). *The dance language and orientation of bees.*

Wang, L., Elliott, B., Jin, X., Zeng, L., & Chen, J. (2015). Antimicrobial properties of nest volatiles in red imported fire ants, Solenopsis invicta (hymenoptera: Formicidae). *The Science of Nature*, *102*(11), 66. https://doi.org/10.1007/s00114-015-1316-1

Wang, X.-L., & Yao, Y.-J. (2011). Host insect species of Ophiocordyceps sinensis: A review. *ZooKeys*, *127*, 43–59. https://doi.org/10.3897/zookeys.127.802

Way, M. J., & Khoo, K. C. (1989). Relationships between Helopeltis theobromae damage and ants with special reference to Malaysian cocoa smallholdings. *Journal of Plant Protection in the Tropics*, *6*(1), 1–11.

Way, M., & Khoo, K. (1992). Role of ants in pest management. *Annual Review of Entomology*, *37*(1), 479–503.

Weber, N. A. (1957). The nest of an anomalous colony of the arboreal ant Cephalotes atratus. *Psyche: A Journal of Entomology*, *64*(2), 60–69.

Weinstein, S. B., Moura, C. W., Mendez, J. F., & Lafferty, K. D. (2018). Fear of feces? Tradeoffs between disease risk and foraging drive animal activity around raccoon latrines. *Oikos*, n/a-n/a. https://doi.org/10.1111/oik.04866

Wesołowska, W., & Wesołowski, T. (2014). Do Leucochloridium sporocysts manipulate the behaviour of their snail hosts? *Journal of Zoology*, *292*(3), 151–155. https://doi.org/10.1111/jzo.12094

Wetterer, J. K. (2017). Geographic distribution of the weaver ant Oecophylla smaragdina. *Asian Myrmecology*, *9*, 1–12.

Wilensky, U. (1999). *NetLogo*. Center for Connected Learning and Computer-Based Modeling.

Wilson, E. O. (1971). *The insect societies.* Cambridge, Massachusetts, USA, Harvard University Press.

Wilson, Edward O. (2000). *Sociobiology: The new synthesis*. Harvard University Press.

Wilson, K., Bjørnstad, O., Dobson, A., Merler, S., Poglayen, G., Randolph, S., Read, A., & Skorping, A. (2002). Heterogeneities in macroparasite infections: Patterns and processes. *The Ecology of Wildlife Diseases*, *44*, 6–44.

Wilson-Rich, N., Spivak, M., Fefferman, N. H., & Starks, P. T. (2009). Genetic, Individual, and Group Facilitation of Disease Resistance in Insect Societies. *Annual Review of Entomology*, *54*(1), 405–423. https://doi.org/10.1146/annurev.ento.53.103106.093301

Wynne, R., Morris, A., & Rae, R. (2016). Behavioural avoidance by slugs and snails of the parasitic nematode Phasmarhabditis hermaphrodita. *Biocontrol Science and Technology*, *26*(8), 1129–1138. https://doi.org/10.1080/09583157.2016.1185513

Yang, L., Zhang, Y., Chen, J., Zhang, S., & Chen, D. Z. (2017). Suggestive Annotation: A Deep Active Learning Framework for Biomedical Image Segmentation. *Medical Image Computing and Computer-Assisted Intervention − MICCAI 2017*, 399–407. https://doi.org/10.1007/978-3-319-66179-7_46

Yanoviak, S. P., & Dudley, R. (2006). The role of visual cues in directed aerial descent of Cephalotes atratus workers (Hymenoptera: Formicidae). *Journal of Experimental Biology*, *209*(9), 1777–1783. https://doi.org/10.1242/jeb.02170

Yanoviak, S. P., Dudley, R., & Kaspari, M. (2005). Directed aerial descent in canopy ants. *Nature*, *433*(7026), 624–626. https://doi.org/10.1038/nature03254

Yanoviak, S. P., Kaspari, M., Dudley, R., & Poinar, G. (2008). Parasite-Induced Fruit Mimicry in a Tropical Canopy Ant. *The American Naturalist*, *171*(4), 536–544. https://doi.org/10.1086/528968

Yanoviak, S. P., Munk, Y., Kaspari, M., & Dudley, R. (2010). Aerial manoeuvrability in wingless gliding ants (Cephalotes atratus). *Proceedings of the Royal Society of London B: Biological Sciences*, *277*(1691), 2199–2204. https://doi.org/10.1098/rspb.2010.0170

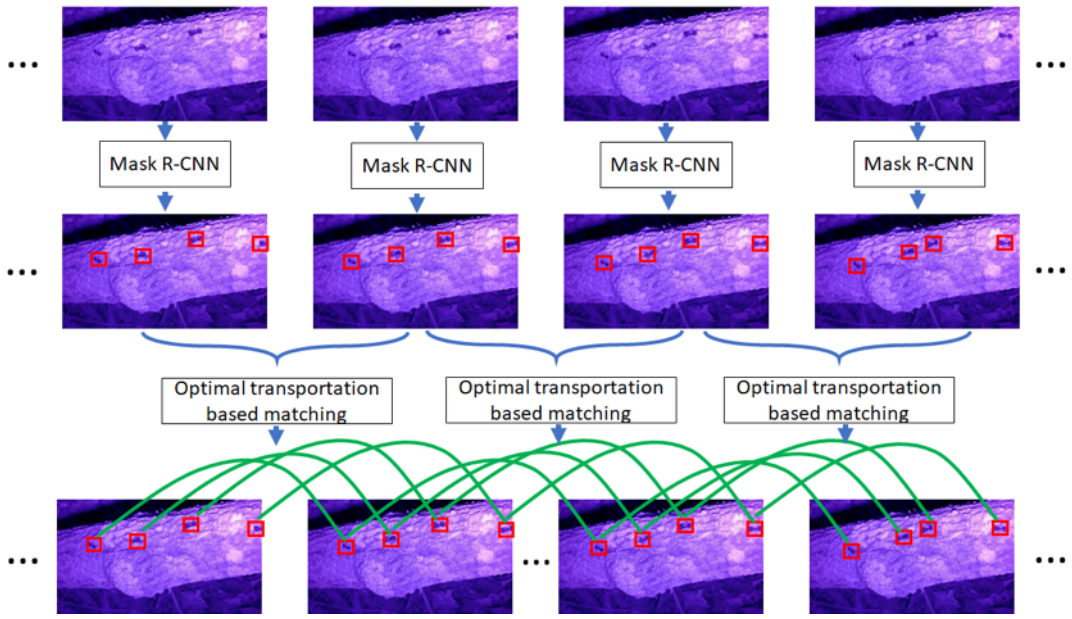
Zeileis, A., Cribari-Neto, F., Gruen, B., Kosmidis, I., Simas, A. B., Rocha, A. V., & Zeileis, M. A. (2016). Package 'betareg.' *R Package*.

Appendix A

Supplementary Material for Chapter 2

Description of the ant detection and tracking approach

The overall pipeline of our approach for ant detection and tracking is sketched in the following figure. We first apply a Mask R-CNN model [2] on every image frame in a video to detect all ants (and their positions) in the frame; we then apply an optimal transportation based tracking method [1] to match and connect the detected ants in each frame to form ant trajectories for individual ants throughout the video.



The Mask R-CNN model:

Overall, we hand-labeled 20666 images for training a Mask R-CNN model [2]. Our code for training and testing the Mask R-CNN model can be found via the Google Drive link below: https://drive.google.com/drive/folders/15w3VAhG9vvc0-Psx2cjoEpBh8EFy4rTE?usp=sharing

Optimal transportation based ant matching/tracking method:

We applied the tracking method developed in [1] for tracking ants detected by the Mask R-CNN model. Our code for this method can be found via the Google Drive link below: https://drive.google.com/drive/folders/19REUh1HmD97niFB4KlkZ8QxeqR6dRW6h?usp=sharing

[1] J. Chen, C. W. Harvey, M. Alber, and D. Z. Chen. A matching model based on earth mover's distance for tracking Myxococcus xanthus. In *International Conference on Medical Image Computing and Computer-Assisted Intervention (MICCAI)*, pp. 113-120, 2014.

[2] K. He, G. Gkioxari, P. Dollar, and R. Girshick. Mask R-CNN. *IEEE International Conference on Computer Vision (ICCV)*, pp. 2980-2988, 2017.

Ant visiting map

Trajectories can be embedded into the 2D space of the images in the video, as follows. Suppose we divide an image into a grid structure of size *m x n* (i.e., the image consists of *m x n* grid cells). Then we can represent an ant trajectory *T* as a sequence of grid cells that *T* travels or visits. Further, for each grid cell *C(i, j),* we can count the number *V(i, j)* of times that *C(i, j)* is visited by the ant trajectories. This will generate a map of the image which has a similar effect as a heat map: The larger the value *V(i, j)* is (i.e., the more often the cell *C(i, j)* is visited by the ant trajectories), a higher temperature the cell *C(i, j)* has.

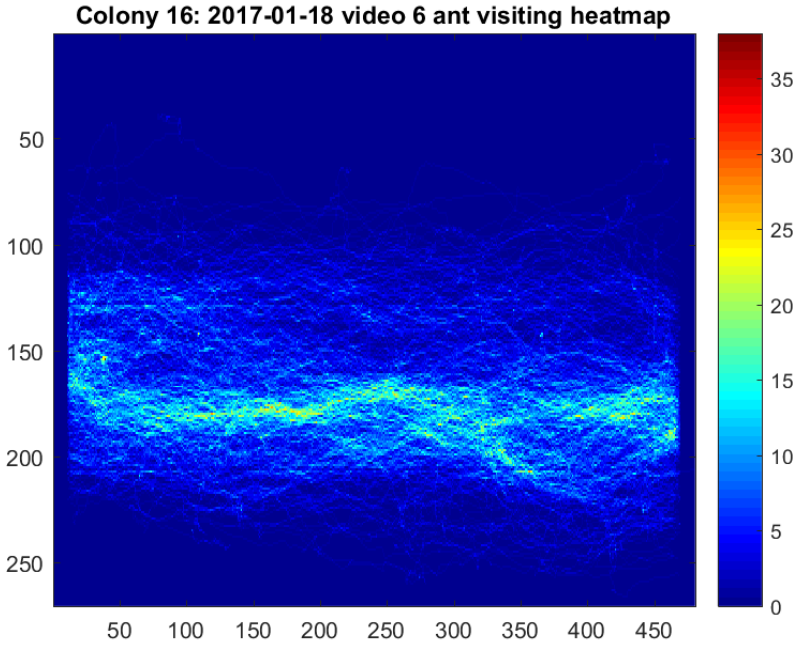
Suppose we have K trajectories $T_1, T_2, \ldots, T_K$. For a trajectory T_k, it consists of a sequence of grid cells $C(x^k_1, y^k_1), C(x^k_2, y^k_2), \ldots, C(x^k_{Pk}, y^k_{Pk})$, where P_k is the number of cells in trajectory T_k.

We compute the “ants visiting map" of trajectories using the following procedure.

Algorithm 1 Compute Ants Visiting Map

```
function ANTS VISITING MAP(T_k, k = 1, 2, ..., K)
    Create an empty array V of size m × n
    for k ← 1 to K do
        P_k ← the number of cells in T_k
        for p ← 1 to P_k do
            V(x_p^k, y_p^k) = V(x_p^k, y_p^k) + 1
    return V;
```

The following is an example image output from the above calculation:



Exploration index

After computing the ants visit map, we can further compute an index, called the exploration index (EI), of each trajectory. The exploration index may better capture the ant exploration of the image areas. For every grid cell *C(i,j)*, we give it an initial exploration value *E(i,j)*. To avoid introducing any bias here, we set *E(I,j)* as a constant (e.g.,1) for every grid cell *C(i,j)*. Then when a trajectory *T* passes through cell *C(i,j)*, it picks up a value *E(i,j)/V(i,j)*, where *V(i,j)* is the number of trajectories that visit *C(i,j)* computed in the above procedure. That is, the more trajectories pass through cell *C(i,j)*, a lower value of exploration that the trajectories will pick up from visiting *C(i,j)* (as *C(i,j)* is visited by multiple ants, its exploration value becomes low. We may then compute the total sum of exploration values in all the cells of trajectory *T* as the exploration index (EI) of *T,* and also the average exploration index (AEI) of *T* which can be obtained by dividing the EI by the length of the trajectory. When comparing between videos in a night of footage, we removed short videos or early videos with only a few trajectories as this gives inaccurate exploration indices.

Figure AA-1. Image of field camera set-up for filming ants on trails.

GoPros were attached to a 30cm clamp that was attached to a pole placed in a ground 30cm from the trail. An infrared light was attached the end of the clamp next to the GoPro and powered by a 12-volt battery (pictured to the left). The camera was approximately 30 cm above the trail.

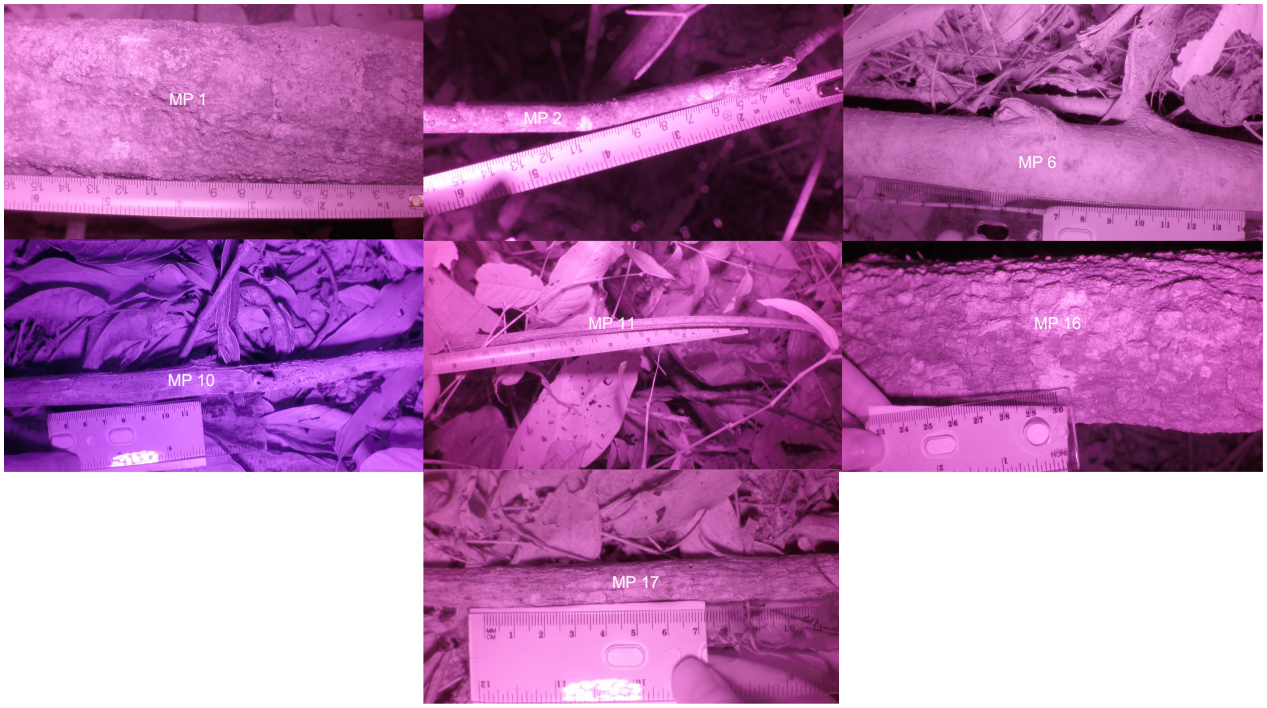


Figure AA-2. Images of trunk trails filmed for all colonies.

Images taken from GoPro footage, pink/purple color due to infrared light and infrared filter in camera.

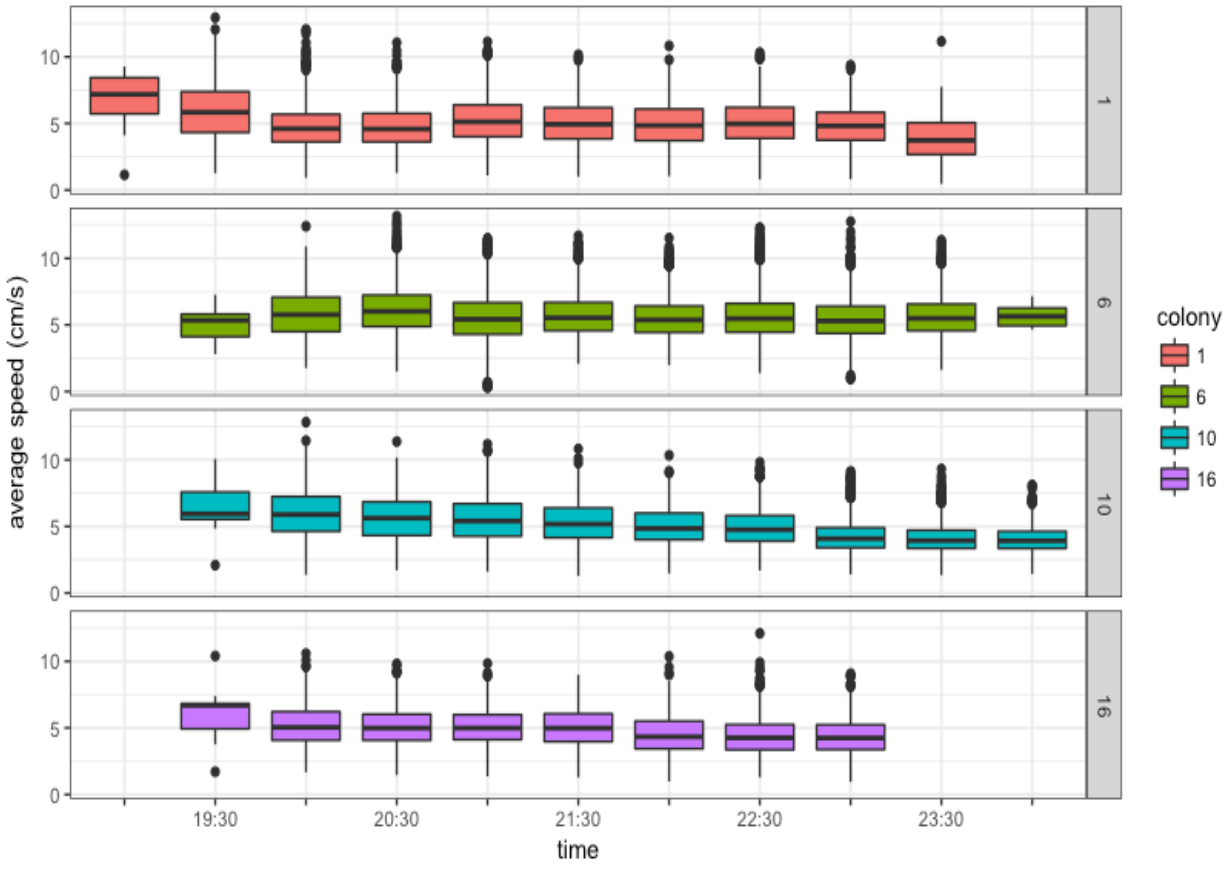


Figure AA-3. Speed of ants over time.

Average speed of ants within a 30-minute interval, broken down by colony.

Table A-1. Summary of field studies that have observed ant foraging trails.

Method of collecting data	Observer of behavior	Alterations to environment	Type of trail?	Reference
in person	human	none	trunk	(Gordon, 2013)
in person	human	none	trunk	(Gordon, 1991)
film	human	none	raid	(Couzin & Franks, 2003)
film	human	artificial surface	trunk	(Burd & Aranwela, 2003)
in person	human	column around trail	trunk	(Moffett, 1987)
in person	human	none	trunk	(Anderson & Jadin, 2001)
film	computer	ants walked on index card	temporary	(Pearce-Duvet et al., 2011)
in person	human	baiting	temporary	(Lynch et al., 1980)
in person	human	mark-recapture	trunk	(Porter & Jorgensen, 1981)
in person	human	trail disturbance	trunk	(Evison et al., 2008)
in person	human	feeding sites, gut contents used to create artificial trails	temporary	(Hölldobler, 1981)
in person	human	gridlines; feeding sites	temporary	(Kohler & Wehner, 2005)
in person	human	baiting	temporary	(Holway & Case, 2000)
in person	human	none	trunk	(Heller & Gordon, 2006)
film/in person	human	baiting	temporary/trunk	(Flanagan et al., 2013)
in person	human	removed ants	trunk	(Gordon et al., 2011)
film	human	walked on masonite board in respirometer chamber	trunk	(Lighton & Duncan, 2002)
in person	human	removed ants	extrafloral nectary visitation	(Dreisig, 2000)
film	human	removed bridges & inserted specially made apparatus	raid	(Reid et al., 2012)
in person	human	feeders	temporary	(Latty & Beekman, 2013)
in person	human	none	temporary	(Detrain, 1990)
in person	human	none	trunk	(Detrain et al., 2000)

Copy of published paper

www.nature.com/scientificreports

SCIENTIFIC REPORTS
nature research

OPEN

Automated tracking and analysis of ant trajectories shows variation in forager exploration

Received: 6 November 2018
Accepted: 29 August 2019
Published online: 13 September 2019

Natalie Imirzian[1], Yizhe Zhang[2], Christoph Kurze[1,3], Raquel G. Loreto[1,4], Danny Z. Chen[2] & David P. Hughes[1,3]

Determining how ant colonies optimize foraging while mitigating pathogen and predator risks provides insight into how the ants have achieved ecological success. Ants must respond to changing resource conditions, but exploration comes at a cost of higher potential exposure to threats. Fungal infected cadavers surround the main foraging trails of the carpenter ant *Camponotus rufipes*, offering a system to study how foragers behave given the persistent occurrence of disease threats. Studies on social insect foraging behavior typically require many hours of human labor due to the high density of individuals. To overcome this, we developed deep learning based computer vision algorithms to track foraging ants, frame-by-frame, from video footage shot under the natural conditions of a tropical forest floor at night. We found that most foragers walk in straight lines overlapping the same areas as other ants, but there is a subset of foragers with greater exploration. Consistency in walking behavior may protect most ants from infection, while foragers that explore unique portions of the trail may be more likely to encounter fungal spores implying a trade-off between resource discovery and risk avoidance.

Resource acquisition drives animals into new territories, while threat avoidance limits where animals move. A consistent threat is the presence of infectious propagules of parasites and these are hypothesized to be major determinants of the distribution of animals in the wild[1]. Examples of animals avoiding pathogen contaminated areas span diverse taxa, from mammals to insects, implying anti-parasite behavior is widespread[1–5]. Central place foragers are interesting in the context of parasite avoidance as they must obtain food while avoiding threats with the additional constraint of returning to a defined location after each trip. For volant central place foragers, like wasps, bees, bats and birds, much of the trip is through the air likely reducing contact with infectious material. However, for taxa which walk on the ground (e.g. ants), encounters with parasite propagules are presumably higher[6]. Unlike threats from mobile predators and competitors, parasites could directly alter movement patterns since infection occurs from a stable location on the ground. For social organisms, it would be advantageous to avoid pathogen contaminated areas in order to protect the entire colony from becoming infected.

While some ant species send workers out from the colony to forage independently, other ant species use highly coordinated groups to forage, often facilitated through chemical signaling[7]. Group foraging via chemical trails can lead to semi-permanent trails known as 'trunk trails'[8]. Trunk trails stimulate research interest largely from the perspective of the self-organization behavior of ants, such as how ants regulate traffic[9–11]. Trunk trails have also been studied from the perspective of their temporal and spatial dynamics as well as their energetic value in terms of efforts expended and resources obtained[12,13]. Yet, studies have not investigated how utilizing the same trails day after day impacts the exposure of ants to threats. Moreover, studies on ant foraging have largely occurred in a laboratory setting, and of the work that took place in the field, most studies relied on human observation or manipulated the environment in some way (see references in Supplementary Table S1). An ant species that forages collectively and predictably in time and space would be useful to assess the relationship between trail behavior and risk avoidance.

A potential system is the carpenter ant *Camponotus rufipes* in southeastern Brazil, which forms trunk trails lasting for multiple months[14,15]. Colonies of this ant were recorded as having a chronic infection by the fungal parasite *Ophiocordyceps camponoti-rufipides* across 20 months[16,17]. This fungus manipulates foragers to leave the nest

[1]Department of Entomology, Pennsylvania State University, University Park, PA, USA. [2]Department of Computer Science & Engineering, University of Notre Dame, Notre Dame, IN, USA. [3]Department of Biology, Pennsylvania State University, University Park, PA, USA. [4]Department of Entomology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. Correspondence and requests for materials should be addressed to N.I. (email: nsi2@psu.edu)

and die biting the underside of a leaf[17,18]. To complete its lifecycle, the fungus must grow out of the ant cadaver and form a fruiting body that releases spores onto the ground below that will infect other ants[18]. Cadavers are found attached to leaves surrounding the ant nest[17]. The chronic nature of infection at the colony level means the spores of the pathogen are continuously in the environment from the perspective of the foragers. The spores are curved and large (80–95 microns)[16] implying they do not travel far and land on the nearby trails once released from ant cadavers that hang above trails. Spores germinate to produce infectious secondary spores on hairs (capilliconidia) which attach to ants as they walk over them[19]. Thus, infection does not require a spore to hit an ant as it walks on a trail below a cadaver. Instead, the trail substrate itself serves as the source of contamination.

Foragers of the carpenter ant *C. rufipes* mostly collect nectar from hemipteran secretions and extrafloral sources[14,20]. The exploitation of a stable resource suggests that the most efficient way for a colony to obtain resources is for the majority of foragers to walk directly to the food source, utilizing trails near the colony entrance as a highway. However, if all foragers walked directly towards the food source, this would hinder the colony's response to changes in resource availability. We hypothesize that some individual trajectories will show evidence of searching behavior, but the majority of ants will walk directly across the trail and cover similar areas limiting the exposure of most ants to threats.

We studied the trails of seven *C. rufipes* colonies in their rainforest habitat to determine how individual ant trajectories vary in their consistency and coverage of trail space to investigate whether all foragers are at equal risk of encountering a fungal spore. Importantly, we studied ant movement on undisturbed trails, keeping pathogen risk at natural levels and including the factors undetectable to humans that influence ant foraging. We devised a system of recording trails using infrared lights and modified cameras to contend with the nocturnal foraging of this species. We then used computer vision and deep learning to automate ant tracking then characterized forager trajectories on speed, straightness, direction, and exploration.

First, we focused on the straightness of trajectories to assess the efficiency of the colony in food retrieval and to investigate whether some ants are engaged in searching behavior. Next, we analyzed the tendency of trajectories to cover unique areas of the trail through calculation of an "exploration index" of each trajectory. We predicted that most trajectories will have high straightness and low exploration scores as this increases food retrieval while limiting risk exposure. We then investigated the relationship between straightness and exploration, as well as exploration and time. We predicted that ants that walk straight across the trail are more likely to cover the same area of the trail as other ants (low exploration), while ants with lower straightness scores are more likely to walk over a new area of the trail (high exploration). We also predicted exploration levels would be higher at the beginning of a foraging period, as this is when the pheromone trail would be the weakest. We found that some ants wander when crossing the trail and these ants are more likely to explore a unique area of the trail, possibly increasing the flexibility of the foraging system by heightening food discovery. Conversely, covering a new area of the trail could expose wandering ants to threats other ants may avoid through following the main foraging trail.

Methods

Study site. Fieldwork took place at the Research Station of Mata do Paraíso, Universidade Federal de Viçosa, Minas Gerais, Southeast Brazil (20°48′08S 42°52′31W) between 10 and 25 January 2017. The carpenter ant *Camponotus rufipes* is abundant in this area, forming trails lasting multiple months[14,15]. The forest floor in the area of study is usually covered in 10–20 cm of leaf litter. Instead of traversing through the leaf litter, *C. rufipes* trails often use 'bridges' composed of woody debris, lianas, and tree branches 2 cm or more above the leaf litter[15]. Occasionally, when there are patches of clear soil (usually due to human made paths) trails would cross these areas. Ants forage at night and activity peaks in the early evening.

Trail filming. Trails from seven different *C. rufipes* nests were filmed between 10 and 25 January 2017. Nests were selected based on their location and structure. Only nests found above the ground with nest material clearly visible were used. Trails were filmed before a branching point from the main trail so that ants were filmed coming directly from or towards the nest. In the case where multiple trails came from one nest, the busiest trails were selected. The width of the branches filmed ranged from 0.8 cm to 7 cm (mean ± standard deviation; 2.97 cm ± 2.53) and the length of the area filmed for all branches was approximately 15 cm.

GoPro cameras (model: HERO 3+, GoPro, Inc., San Mateo, USA) with a modified infrared filter (RageCams.com, Michigan, USA) were used for filming. Stakes were placed 30 centimeters from the trails and 30 cm medium trigger clamps (DWHT83140, DeWalt, Towson, USA) were attached to the stakes. Cameras were attached to clamps so that cameras were approximately 30 centimeters above the trails looking down at the ants walking on the trails (Supplementary Fig. S1). An additional camera was placed on the stake, looking sideways at the ants, to allow another perspective for behavioral analysis. Filming lasted from 19:30 to 00:00 for 4–7 nights for each trail (Supplementary Table S2). Timing of filming was based on previous work showing activity begins around 19:30 and peaks around 21:00[15]. Infrared lights (IR30, CMVision, Houston, USA) were connected 12-Volt 7Ah batteries (UP1270, UniPower, São Paulo, Brazil) to allow illumination of the trail without disturbing the behavior of the ants. The camera batteries lasted for approximately 1.5 hours, so the battery was changed once in the middle of a filming period. Slight adjustments in where the trail was positioned in the video view would sometimes occur at this time. Figure 1a shows an example image of a trail filmed and images of the remaining trails filmed are found in Supplementary Fig. S2.

Automated ant tracking. A total of 78 hours and 56 minutes of video were recorded for seven colonies across four nights (Supplementary Table S2). We developed a machine learning approach to process and analyze these videos using a deep learning based segmentation model that identified ants as they came onto the screen and tracked them as they moved across the screen (Supplementary Material).

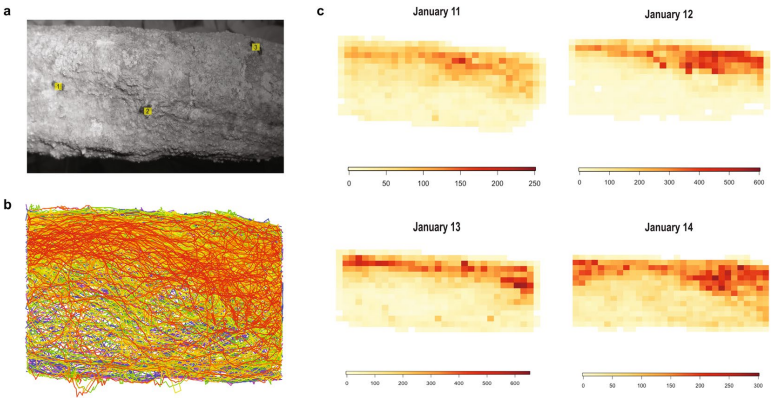


Figure 1. Trail image, trajectory overlay, and collective movement pattern. (**a**) Example trail image from GoPro footage of colony MP1. Individual ants are labeled with identification numbers. (**b**) All of the trajectories from a single night of footage (January 14) at colony MP1. Each line across the trail represents a different ant, with the different colors distinguishing between different ant tracks. (**c**) The trail space from (**a**) was divided into a grid with each square representing approximately 1 cm^2. The number of times an ant walks into a square of the grid was calculated and the darker colors represent areas of the trail that ants walked over more. Each heatmap represents a different date (January 11 through January 14) from approximately the middle of the night to control for differences in the timing of filming. Different scales were used for each night, due to variance in the number of ants that walked across the trail.

Our automatic ant tracking method contains two main processes: (1) detecting ants in each image frame of all videos, and (2) building ant trajectories for every video based on the detected ants. Commonly, deep learning schemes require a large amount of labeled ground truth data for model training. Since our dataset is quite large (>8 million image frames), we aimed to generate sufficient labeled data for training our deep learning model without incurring excessive human labeling effort. Also due to the large size of our dataset, common active learning based sample selection methods (e.g.[21]) are not efficient. The goal of ant detection is to build ant movement trajectories and since ant trajectories normally span multiple consecutive frames in videos, detected ant positions in earlier frames assist with ant detection in later consecutive frames. That is, while ant detection forms a basis for building ant trajectories, trajectories of detected ants may also help ant detection. Hence, we designed our trajectory building procedure such that it not only can track detected ants but also can provide cues to indicate where (which frames and locations) there might be inconsistencies in ant trajectories and difficult scenarios for ant detection (e.g. densely clustered ants). We used such cues to select difficult cases from the frames for labeling to improve the deep learning detection model as well as the ant detection results. Therefore, our detection-tracking method consists of two rounds (with the second round improving the detection and tracking results of the first round), and each round performs two major steps, ant detection and trajectory building, as described below.

Ant detection. This aims to detect ants in all the frames of the videos. We applied a novel object detection and segmentation model, Mask R-CNN[22], to automatically detect ants in every frame.

Ant trajectory building. Given the detected ants in each frame, the next step is to form ant trajectories that connect detected ants frame-by-frame in videos. We formulated this ant trajectory building problem as a *transportation problem*, that is, between every two consecutive frames in each video, we find an optimal transportation (for ants) that corresponds to real movement of ants. In this transportation formulation, each detected ant in frame K can be viewed as a 'supplier' and each detected ant in frame $K+1$ can be viewed as a 'receiver'. The dissimilarity (based on spatial distance and appearance difference) between ants in two consecutive frames is a measure of how much 'cost' it would take to transport (move) one ant in frame K to another in frame $K+1$. The objective is to transport detected ants (as many as possible) in frame K to frame $K+1$ with the minimum total cost. Optimal transportation based tracking methods are known to be effective for tracking sets of moving and changing objects in image sequences[23,24].

In the first round, we randomly selected frames to label as training data. This allowed us to quickly and unbiasedly obtain data samples for training a decent detection model. We then applied the trained model to all of the frames to produce ant detection results. We conducted trajectory building on detected ants to form the ant trajectories. Besides tracking ant movement, our trajectory building procedure in the first round also provided cues for identifying inconsistencies in ant trajectories and difficult cases in the frames for ant detection. In the second round, we applied training data selection to those difficult cases to find additional frames for labeling, and the enlarged training dataset thus obtained was used to re-train the Mask R-CNN detection model. The re-trained

detection model was then applied to all the frames to produce the final ant detection results, which were used to build the final ant trajectories in the videos.

To identify difficult cases for additional training data selection, we used the following set of measures to capture possible errors in ant detection and trajectory results. (i) Ant speed: At a place where ants usually do not move very fast but a fast movement is suggested by the optimal transportation solution, this instance might indicate an error in ant detection. (ii) Missing ants in the middle part of a tree branch: When the optimal transportation solution does not find a corresponding ant instance in the next frame in the interior section of a tree branch, it might suggest a missing data point in ant detection. (iii) Ant identification (ID) switching: Each detected ant was assigned an ID number; when multiple ants are seen at spatially close interaction and slight changes on the dissimilarity scores among these ants give largely different solutions for the optimal transportation problem, this might suggest an ant ID switch error. Based on these observations and measures, our trajectory building process can help identify difficult detection and tracking cases for additional training data selection to improve model performance.

Overall, we annotated 20,666 images for training the deep learning model for the ant detection task. Thus, the model is fairly robust to complex backgrounds, low contrast image areas, illumination differences. Besides relying on the training data and the robustness of Mask-RCNN model, our tracking algorithm works on the temporal information and is also robust to false-detection and miss-detection of ant. In particular, our tracking algorithm is tuned to be very robust to false-positive detections. Namely, our tracking algorithm has a strong prior/preference to discarding false-detections using temporal information. When we train and apply the Mask-RCNN model, we tolerate the Mask-RCNN model to produce some false-positive detections in order to keep the number of miss detections very low. For occasional miss-detection cases, our tracking algorithm can also recover them using temporal information.

Our automatic ant detection and tracking method extracted the x and y coordinates in pixels of detected ants in every frame and assigned each ant an identification number (Fig. 1a and Supplementary Video S1). Ant identification numbers were used to form ant trajectories used in further analysis.

Error assessment. To assess the accuracy of the computer model, we watched a subset of videos and determined the error rate. GoPro cameras automatically divide footage into 26-minute-long videos, so one night of footage at a single trail has 6 to 10 videos. This provides a way of checking the accuracy of the computer tracking at random points throughout a night. We first error checked videos from the middle of the night (when the trails should be busiest) to determine if the data from that colony was high enough quality to use in our analysis. If the average accuracy was greater than 60% for these videos, we continued to error check all videos and nights for that colony. To error check, we counted the number of ant trajectories with errors out of the first 15–30 tracked ants. The number of ant trajectories checked varied because videos from early in the foraging period sometimes had fewer ants.

To ensure consistency in the type of ant trajectories that were analyzed, trajectories beginning in the middle of the field of video view were removed. This created uniformity between all colonies and nights in the type of ants that were compared as it focused on the ants that made it from one end of the trail to the other completely in the view of the video.

Trajectory analysis. We used R version 3.4.4 and RStudio version 1.1.447 for all analyses[25,26]. Ant location data was frame-by-frame, so we used the native frame rate of the cameras (29.97 or 25 frames per second; the default setting of the cameras varied) to convert the time in frames to seconds and then used the start times of each video to convert it to real time (Supplementary Table S2). To convert ant location data from pixels to centimeters, we placed a ruler in each video to determine the conversion factor (Supplementary Fig. 2).

To determine how individual ants were moving, we calculated the following variables: average speed, overall direction, and straightness. Average speed was taken as the total distance an ant travels while in the video over the time it takes for them to travel that distance. Overall direction was whether the ant headed away from or towards the nest which we determined based on where the ant entered and exited the video view. A variety of measures are used to determine the straightness or tortuosity of an animal's movement path[27,28]. Ant movement on trunk trails is expected to move in an oriented direction, and not be a random search path, thus we used the simplest measure, the straightness index[28]. The straightness index (ST) is a ratio between the net displacement and total path length:

$$ST = d/L;$$

where d = the distance between the beginning and end of the path and L = total path length.

To assess similarity between individual ant trajectories, we calculated an exploration index (EI) for each trajectory (Supplementary Information). The exploration index measures how much an individual trajectory covers unique areas of the trail space. First, we computed an Ants Visiting Map (Supplementary Information) for a video which estimates how frequently ants are visiting different parts of the trail. We then scored grid cells of the trail space based on how many trajectories pass through each cell. The exploration index for an individual trajectory is calculated from the scores of the grid cells that the trajectory passes through. If a trajectory mostly passes through areas of the trail space that are visited by many ants, the individual trajectory will have a low EI. To control for trajectory length, we divided the EI for a trajectory by trajectory length to get an average exploration index (AEI) for each trajectory.

Inspection of the trajectories showed that some ants performed U-turns, where they would exit the field of view from the same side that they entered on (Supplementary Video S2). To more accurately represent the shape of the trajectories, we broke U-turning trajectories into two parts at the point the trajectory turned from one direction on the trail to the other and calculated straightness and exploration for the different trajectory parts individually.

Statistical analysis. A linear mixed-effects model was used to assess whether the speed of ants changes over a foraging period. The model was generated using the lmer function in the R package' lme4'[29], with speed as the response variable, time as the fixed effect, and colony and date as the random effects. The package 'lmerTest'[30] was used to generate p-values. We checked the plotted residuals to ensure homoscedasticity prior to utilizing the results of the model. We also used a linear mixed-effects model to test whether the trajectories of ants with lower straightness scores have higher exploration values. We included colony, date, and video as random effects. We fit our model with the straightness index (ST) as the fixed effect and the response variable as the average exploration index (AEI).

To analyze whether exploration differed across a foraging period, we compared the average exploration index within 30-minute intervals across the recording period. We pooled our data within 30-minute intervals to overcome discrepancies in recording times across dates. We fit a linear mixed-effects model with the interval as the fixed effect, colony and date as random effects, and the AEI for that interval as the response variable. We used a comparison of means with the Tukey method to investigate how the AEI of trajectories differed between 30-minute intervals.

Results

Automated tracking performance. The automated tracking of ants in video frames resulted in 20,230,585 data points on ant movement. The model had two types of accuracy against which it can be judged, relative to a human. The first is species accuracy (detection accuracy) which is a measure of how well the model recognized the correct species of ant. The model correctly detected *C. rufipes* ants with an accuracy of 97.86%. The model picked up other insects or species of ants on the trail (false positive) or failed to detect a *C. rufipes* ant as it went across the trail 2.14% of the time.

The second accuracy measurement is tracking accuracy. The computer had to detect *C. rufipes* ants and follow them as they moved across the screen. If an ant moved in a straight line this required the computer to recognize and track that ant for about 4 seconds or 120 frames. The computer assigned identification numbers to individual ants to follow an ant as it travelled across the screen. The machine learning model sometimes made errors in doing this. The computer may switch identification numbers when ants walked too closely together (Supplementary Video S3). An average of 78.70% of complete ant trajectories across all colonies had no mistakes as identified by a human observer (Supplementary Table S3). The tracking accuracy was the lowest for colonies MP2 (40.0%), MP11 (31.7%), and MP17 (50.6%). Identification number switches commonly happened in colonies MP2 and MP11. These trails were very thin and introduced more challenges in determining the trajectories of individual ants, so they were removed from further analysis. We have additionally removed colony MP17 as an obstruction in the trail led to ants departing from the branch and walking underneath leaves (Supplementary Video S4). Ants disappearing under leaf debris made it difficult to track an individual ant. We have made all videos and data available as we expect improved future machine learning models can make use of them.

The exclusion of these colonies brought the size of the dataset to 8,412,477 data points on ant movement from four colonies: MP1, MP6, MP10, and MP16. The large reduction in number of data points from the elimination of 3 colonies can be attributed to the configuration of these trails creating congested areas on the trails where single ants were tracked multiple times falsely inflating the number of ants and overall data points. The data points from the 4 included colonies represents the movement data for 64,498 ants. The average tracking accuracy of the remaining colonies was 81.39% (MP1: 72.0%; MP6: 82.1%; MP10: 77.2%; MP16: 92.1%). Most errors were due to an identification number switching to a different ant (8.28%). The high error rate for colony MP1 could be attributed to the darkness of the videos causing the model to miss part of an ant's trajectory or failing to detect an ant in the dark areas of the trail. If we consider only the errors where a number is on a wrong ant or a number is not on an ant, the accuracy improves greatly (overall: 90.94%; MP1: 91.5%; MP6: 88.8%; MP10: 86.6%; MP16: 96.3%). We are mainly concerned with the direction and shape of trajectories, and the main error that impacts an individual ant's trajectory is when ants switch to the wrong identification number, so the second calculation of accuracy rate is more reflective of this.

Collective movement pattern. Most ants walk on the same area of the available trail space (Fig. 1). The trail usage pattern is consistent between nights (Fig. 1c). The mean speed of all ants from all colonies and nights was 5.15 cm/s ± 1.63 (standard deviation). The average speed of the colonies ranged from 4.74 cm/s to 5.62 cm/s and within colony variability in speed was similar between colonies (mean (cm/s) ± standard deviation; MP1: 4.94 ± 1.72; MP6: 5.58 ± 1.62; MP10: 4.82 ± 1.55; MP16: 4.72 ± 1.43). The results of the linear mixed effects model showed that ant speed decreases by 0.45 cm/s ± 0.07 (standard error) throughout the night ($t_{(94)} = -6.60$, $p < 0.0001$) (Supplementary Fig. S3).

Individual trajectory analysis. Most ants walked in nearly straight lines (Fig. 2a). However, the negative skew of the distribution highlights the tendency of ants with low straightness scores to wander across the trail (Fig. 2a,b and Supplementary Video S5). The median straightness score across all colonies was 0.88 and was similar for each colony (MP1: 0.87; MP6: 0.89; MP10: 0.86; MP16: 0.87). We fit a beta mixture model using the R package betareg[31] to determine whether the distribution represents different groups. We used the Bayesian Information Criterion (BIC) to assess model fit and found that the distribution was best represented by four groups: straight (37.0%; n = 25,224; mean straightness = 0.94), semi-straight (26.2%; n = 17,840; mean straightness = 0.88), semi-curvy (30.0%; n = 20,437; mean straightness = 0.77), and curvy (6.8%; n = 4623; mean straightness = 0.49). The semi-curvy straightness group has a minimum straightness score of 0.64, so 93.2% of ants have straightness scores greater than 0.64.

The distributions of average exploration index (AEI) of trajectories differed in shape for each colony (Fig. 3). Across all colonies, a majority of ants showed low levels of exploration, but the positive skew of the AEI

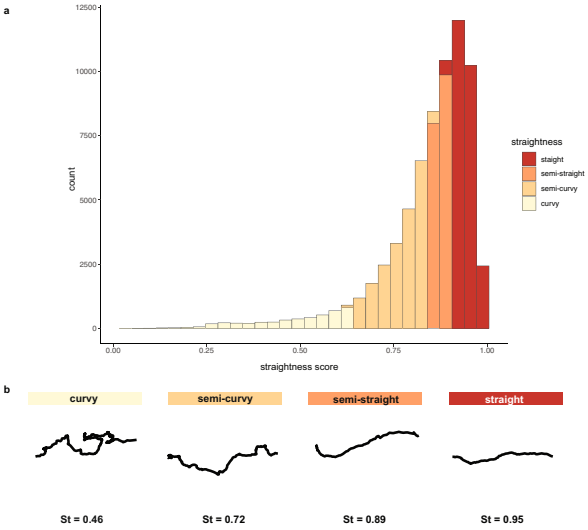


Figure 2. Straightness of trajectories. (**a**) Histogram showing the distribution of straightness scores of ant trajectories for all nights and colonies. (**b**) Example trajectories for ants with different straightness scores. The straightness score (St) for each trajectory is included below. All 4 example trajectories were taken from the same colony and night (colony MP16 – January 15). Supplementary Video S5 features video of the example ants with their trajectories annotated.

distributions indicates a group of ants that are more exploratory (Fig. 3). Colony 1 had the highest median AEI at 0.24, closely followed by colony 16 at 0.19. The median AEI for colony 6 and colony 10 were both approximately 0.06. There was a weak negative relationship between the straightness of a trajectory and its exploration value, as average exploration was estimated to decrease by $0.11 \pm 3.14\text{e-}3$ as straightness increases (linear mixed-effect model; $t_{(6810)} = -36.09$, $p < 2\text{e-}16$). The straightness groups significantly differed in average exploration (Fig. 4a; linear mixed-effect model; $t_{(6810)} = -11.03$, $p < 2\text{e-}16$). Post-hoc analysis using the Tukey Test showed that ants with curvy trajectories had the highest AEI followed by ants with semi-curvy trajectories, then ants with semi-straight trajectories, and ants with straight trajectories had the lowest AEI (linear mixed-effect model; Tukey Test; $p < 0.0001$).

Temporal pattern. Average exploration of trajectories decreased from the beginning of the foraging period to the middle of the foraging period, before increasing slightly again (Fig. 4b). The AEI was significantly greater (linear mixed-effect model; Tukey Test; $p < 0.0001$) at the beginning of the night to all other time intervals. However, the AEI at 22:30 was significantly lower (linear mixed-effect model; Tukey Test; $p < 0.0001$) than at 23:30 or 00:00.

Discussion

Our study used an unobtrusive filming set-up to record behavioral data on more than 64,000 ants moving in a rainforest at night in an area of high disease pressure. Most ants walk in a straight line across the trail, matching our prediction of how ants might behave when using trunk trails (Fig. 2). Similar to straightness, most ants show low levels of exploration, but a subset of ants cover unique areas of the trail (Fig. 3). Average exploration of ants was higher at the beginning of the foraging period (Fig. 4b). Exploration may enhance food discovery, but the low levels of exploration exhibited by the majority of ants may protect most foragers from the risks associated with venturing from the main trail.

The variation in exploration of trajectories indicates that the ants may have different foraging roles. Social insects have members of the colony known as scouts that assist in discovering and recruiting the colony to new food sources[32–35]. The higher exploration levels at the beginning of the night indicate that perhaps some of those ants are acting as scouts and recruiting ants to new food sources. Recruits should subsequently show lower levels of exploration than the scouts as they follow a pheromone trail to the food source. Forager categories can extend beyond just scouts and recruits, as a forager's experience level and information source will alter its behavior[36,37]. A forager recently recruited to a food source must engage in some searching behavior as they follow external stimuli to the food source. Meanwhile, a forager that has already made the trip to a food source is familiar with the route

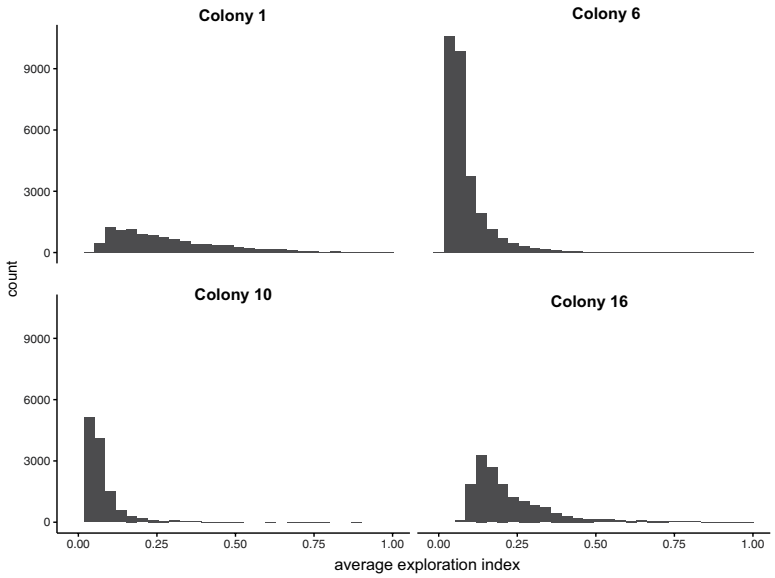


Figure 3. Average exploration of trajectories for different colonies. Histogram showing the distribution of the average exploration index values for all trajectories divided by colony. The average exploration index varies from 0 to 1, with 1 indicating the highest amount of exploration.

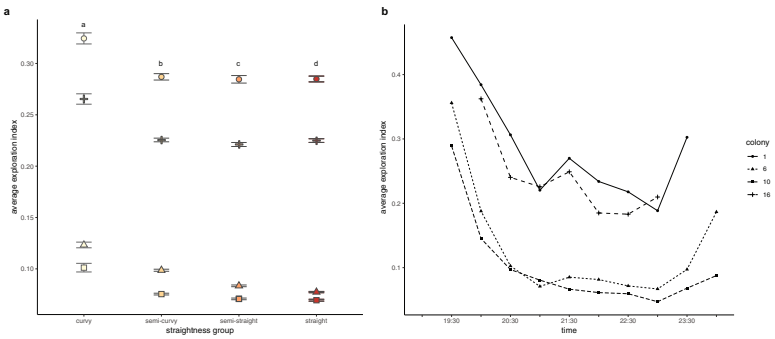


Figure 4. Average exploration across time and for different straightness groups. (**a**) The mean of the average exploration scores for the trajectories in each of the straightness groups from Fig. 2a. Lines indicate ± standard error of the mean. Superscripts indicate straightness groups as significantly different (linear mixed-model $p < 0.0001$). (**b**) The mean of the average exploration scores for all trajectories within each 30-minute interval across the recording period, divided by colony.

and should exhibit less searching behavior. Considering the variation in forager information may explain the distributions of exploration and straightness scores, showing all different levels of straightness and exploration.

A majority of the trajectories likely represent 'employed foragers'[38], or foragers repeatedly exploiting a known resource, since the trails last for multiple months and usually visit a stable homopteran or honeydew secretion. Employed foragers should have lower exploration scores, as their trajectories will overlap other trajectories and this has implications for disease risk. Fungal infected cadavers surround the trunk trails of *Camponotus rufipes* in this habitat, likely dropping spores directly onto the trails below[17]. It is not possible to quantify the abundance

and distribution of micron sized spores on trails in a forest, but the long term tracking of cadaver abundance and the proximity to the trails implies spore presence on the foraging trails[17]. Thus, for most ants, only the first ants walking across the trail after spores have dropped would likely pick up spores. In contrast, ants with higher exploration scores, the "explorers", are constantly more likely to encounter a spore that has not been picked up by a different ant. Through the same logic, an explorative ant has a higher chance of discovering a new food source, demonstrating the benefits of this searching behavior.

We filmed only a small area of the foraging trails, providing a brief snapshot of an ant's behavior. To know whether higher exploration values represented ants that were more likely to wander from the trail and discover new food sources, one would need to follow individual ants for their entire foraging trip, which was beyond the scope of this study. In our study area, exploration values were also impacted by the size of the trail, as ants will have higher overlap (= lower exploration) on narrower trails. The wider trails (colony 1 = 6 cm and colony 16 = 7 cm) had higher median exploration scores than the narrower trails (colony 6 = 3 cm and colony 10 = 1.7 cm). Observing ants beyond one portion of the trunk trails could remove differences between colonies on exploration based on trail size. Trail width still has implications in the context of disease exposure, however, as wider trails offer more substrate for possible spores and perhaps colonies that use larger trails have higher levels of infection.

Following individual ants for their entire foraging trip would also clarify whether individual ants vary in their level of exploration across a foraging trip. Experienced foragers tend to continue exploiting the same food source until it runs out[33]. Moreover, individual ants have been shown to be consistent in their exploratory behavior[39]. The ants with low exploration values appear to be in retrieval mode and thus will likely continue exhibiting the same levels of exploration. Laboratory studies on trail bifurcations provide some evidence on the likelihood of ants to explore away from the main trail. For example, when Argentine ants (*Linepithema humile*) were placed in a maze to a food source, over 80% of the total traffic used the shorter path to the food source in the majority of experiments[40]. Ants selecting a longer path, and ignoring pheromone signals, could represent patrollers or explorers. In a study on Pharaoh's ants (*Monomorium pharaonis*), 30% of the foragers failed to reorient themselves when placed into a trail network without other ants[41]. Perhaps these ants that fail to correctly follow the trail represent another group of foragers and match up with the exploratory group observed in our study.

Beyond food discovery and retrieval, other species of ants provide evidence of more roles within foragers, such as trail maintenance and defense. Ants were observed carrying leaves (Supplementary Video S6), although this could be for nest material and not trail cleaning. Another role could be maintaining the pheromone trail. For example, *Atta sexdens* minims help with the pheromone trail instead of food transport[42]. Ants were observed dragging their gaster on the trail likely depositing trail pheromone (Supplementary Video S7). U-turning ants have been shown to deposit pheromones at a higher rate[43]. Perhaps the main distinction between the groups is not in trail exploration, but in pheromone deposition, with the U-turners serving as the ants that are maintaining the strong chemical signal and allowing most ants to walk directly across the trail.

The different walking styles could also reflect defensive behavior. Smaller workers hitchhike on leaf fragments carried by larger workers in *Atta colombica* leaf-cutting ants, and this likely serves as a defense against parasitoid Phorid flies[44]. Flies, that could possibly be parasitoids, were observed closely following ants on the trail and in some cases appearing to land on the ants which may indicate laying eggs which later become endoparasitoids (Supplementary Video S8). Although the prevalence of parasitoid flies attacking *C. rufipes* is unknown, we have observed adult ants infected by decapitating phorid flies in our study area (Supplementary Video S9). The presence of phorids could directly cause the exploring and U-turning behavior, as ants attempt to avoid flies landing on them. A follow up study could investigate this question of parasite avoidance by directly quantifying how ants behave when phorids are in the environment.

In this study, however, we focused on variability in individual forager trajectories. We found a group of foragers that explores more areas of the trail. Increased exploration increases a forager's chance of encountering a new food resource while simultaneously increasing their exposure to possible risks. The variability in forager behavior provides a possible mechanism for how a colony might mitigate risk through only having a small percentage of foragers exploring out from the safety of the main trail. The scale of our dataset, and ability to collect this data across multiple nights and colonies, increases the reliability and strength of our conclusions. Combining computational advances with behavioral observations provides a technique to investigate the mechanisms of individual movement patterns that influence the distribution of animals in time and space.

Data Availability

The original videos analyzed in this study, along with the full tracking dataset, are accessible through Pennsylvania State University's institutional repository ScholarSphere (https://doi.org/10.26207/q14b-gx36). Information about our process of analyzing the videos and links to the code used can be found in the Supplementary Information.

References

1. Moore, J. *Parasites and the behavior of animals*. (Oxford Univ. Press, 2002).
2. Fouks, B. & Lattorff, H. M. G. Recognition and Avoidance of Contaminated Flowers by Foraging Bumblebees (*Bombus terrestris*). *Plos One* **6**, e26328 (2011).
3. Villani, M. G. *et al*. Use of Radiography and Tunnel Castings for Observing Mole Cricket (Orthoptera: Gryllotalpidae) Behavior in Soil. *Am. Entomol* **48**, 42–50 (2002).
4. Wynne, R., Morris, A. & Rae, R. Behavioural avoidance by slugs and snails of the parasitic nematode Phasmarhabditis hermaphrodita. *Biocontrol Sci. Technol.* **26**, 1129–1138 (2016).
5. Weinstein, S. B., Moura, C. W., Mendez, J. F. & Lafferty, K. D. Fear of feces? Tradeoffs between disease risk and foraging drive animal activity around raccoon latrines. *Oikos* **127**, 927–934 (2018).
6. Boomsma, J., Schmid-Hempel, P. & Hughes, W. Life histories and parasite pressure across the major groups of social insects. In *Insect evolutionary ecology: proceedings of the Royal Entomological Society's 22nd Symposium* 139–175 (CABI Publishing, 2005).
7. Hölldobler, B. & Wilson, E. O. *The ants*. (Springer Verlag, 1990).

8. Edelstein-Keshet, L., Watmough, J. & Ermentrout, G. B. Trail following in ants: individual properties determine population behaviour. *Behav. Ecol. Sociobiol.* **36**, 119–133 (1995).
9. Couzin, I. D. & Franks, N. R. Self-organized lane formation and optimized traffic flow in army ants. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 139–146 (2003).
10. Fourcassié, V., Dussutour, A. & Deneubourg, J.-L. Ant traffic rules. *J. Exp. Biol.* **213**, 2357–2363 (2010).
11. Edelstein-Keshet, L. Simple models for trail-following behaviour; Trunk trails versus individual foragers. *J. Math. Biol.* **32**, 303–328 (1994).
12. Kost, C., Oliveira, E. G. D., Knoch, T. A. & Wirth, R. Spatio-temporal permanence and plasticity of foraging trails in young and mature leaf-cutting ant colonies (Atta spp.). *J. Trop. Ecol.* **21**, 677–688 (2005).
13. Howard, J. J. Costs of trail construction and maintenance in the leaf-cutting ant *Atta columbica*. *Behav. Ecol. Sociobiol.* **49**, 348–356 (2001).
14. Jaffe, K. & Sanchez, C. On the nestmate-recognition system and territorial marking behaviour in the ant *Camponotus rufipes*. *Insectes Sociaux* **31**, 302–315 (1984).
15. Loreto, R. G. *et al*. Foraging ants trade off further for faster: use of natural bridges and trunk trail permanency in carpenter ants. *Naturwissenschaften* **100**, 957–963 (2013).
16. Evans, H. C., Elliot, S. L. & Hughes, D. P. Hidden Diversity Behind the Zombie-Ant Fungus *Ophiocordyceps unilateralis*: Four New Species Described from Carpenter Ants in Minas Gerais, Brazil. *Plos One* **6**, e17024 (2011).
17. Loreto, R. G., Elliot, S. L., Freitas, M. L. R., Pereira, T. M. & Hughes, D. P. Long-Term Disease Dynamics for a Specialized Parasite of Ant Societies: A Field Study. *Plos One* **9**, e103516 (2014).
18. Evans, H. C. & Samson, R. A. Cordyceps species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems II. The Camponotus (Formicinae) complex. *Trans. Br. Mycol. Soc.* **82**, 127–150 (1984).
19. Araújo, J. P. M. & Hughes, D. P. The fungal spore: myrmecophilous *Ophiocordyceps* as a case study. In *The Fungal Community: Its Organization and Role in the Ecosystem*, 4th edn. (eds Dighton, J. & White, JM) 359–367 (CRC Press, USA, 2017).
20. Del-Claro, K. & Oliveira, P. S. Ant-Homoptera Interactions in a Neotropical Savanna: The Honeydew-Producing Treehopper, *Guayaquila xiphias* (Membracidae), and its Associated Ant Fauna on Didymopanax vinosum (Araliaceae) 1. *Biotropica* **31**, 135–144 (1999).
21. Yang, L., Zhang, Y., Chen, J., Zhang, S. & Chen, D. Z. Suggestive Annotation: A Deep Active Learning Framework for Biomedical Image Segmentation. In *Medical Image Computing and Computer-Assisted Intervention − MICCAI 2017* 399–407, https://doi.org/10.1007/978-3-319-66179-7_46 (Springer, Cham, 2017).
22. He, K., Gkioxari, G., Dollár, P. & Girshick, R. Mask R-CNN. In *2017 IEEE International Conference on Computer Vision* (*ICCV*) 2980–2988, https://doi.org/10.1109/ICCV.2017.322 (2017).
23. Chen, J., Harvey, C. W., Alber, M. S. & Chen, D. Z. A Matching Model Based on Earth Mover's Distance for Tracking Myxococcus Xanthus. In *Medical Image Computing and Computer-Assisted Intervention – MICCAI 2014* 113–120, https://doi.org/10.1007/978-3-319-10470-6_15 (Springer, Cham, 2014).
24. Chen, J., Alber, M. S. & Chen, D. Z. A Hybrid Approach for Segmentation and Tracking of Myxococcus Xanthus Swarms. *IEEE Trans. Med. Imaging* **35**, 2074–2084 (2016).
25. R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation for Statistical Computing, 2018).
26. R Studio Team. *R Studio: Integrated Development Environment for R*. (RStudio, Inc., 2016).
27. Benhamou, S. How to reliably estimate the tortuosity of an animal's path:: straightness, sinuosity, or fractal dimension? *J. Theor. Biol.* **229**, 209–220 (2004).
28. Almeida, P. J. A. L., Vieira, M. V., Kajin, M., Forero-Medina, G. & Cerqueira, R. Indices of movement behaviour: conceptual background, effects of scale and location errors. *Zoologia* **27** (2010).
29. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).
30. Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. LmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82** (2017).
31. Zeileis, A. *et al*. Package 'betareg'. *R Package* (2016).
32. Von Frisch, K. The dance language and orientation of bees (1967).
33. Seeley, T. D. Division of labor between scouts and recruits in honeybee foraging. *Behav. Ecol. Sociobiol.* **12**, 253–259 (1983).
34. Howard, J. J., Henneman, L. M., Cronin, G., Fox, J. A. & Hormiga, G. Conditioning of scouts and recruits during foraging by a leaf-cutting ant, *Atta colombica*. *Anim. Behav.* **52**, 299–306 (1996).
35. Crawford, D. L. & Rissing, S. W. Regulation of recruitment by individual scouts in *Formica oreas* Wheeler (Hymenoptera, Formicidae). *Insectes Sociaux* **30**, 177–183 (1983).
36. Biesmeijer, J. C. & de Vries, H. Exploration and exploitation of food sources by social insect colonies: a revision of the scout-recruit concept. *Behav. Ecol. Sociobiol.* **49**, 89–99 (2001).
37. Ravary, F., Lecoutey, E., Kaminski, G., Châline, N. & Jaisson, P. Individual Experience Alone Can Generate Lasting Division of Labor in Ants. *Curr. Biol.* **17**, 1308–1312 (2007).
38. Seeley, T. D. *The wisdom of the hive: the social physiology of honey bee colonies*. (Harvard University Press, 2009).
39. d'Ettorre, P. *et al*. Individual differences in exploratory activity relate to cognitive judgement bias in carpenter ants. *Behav. Processes* **134**, 63–69 (2017).
40. Goss, S., Aron, S., Deneubourg, J. L. & Pasteels, J. M. Self-organized shortcuts in the Argentine ant. *Naturwissenschaften* **76**, 579–581 (1989).
41. Jackson, D. E., Holcombe, M. & Ratnieks, F. L. W. Trail geometry gives polarity to ant foraging networks. *Nature* **432**, 907 (2004).
42. Evison, S. E. F., Hart, A. G. & Jackson, D. E. Minor workers have a major role in the maintenance of leafcutter ant pheromone trails. *Anim. Behav.* **75**, 963–969 (2008).
43. Hart, A. & Jackson, D. E. U-turns on ant pheromone trails. *Curr. Biol.* **16**, R42–R43 (2006).
44. Feener, D. H. & Moss, K. A. G. Defense against parasites by hitchhikers in leaf-cutting ants: a quantitative assessment. *Behav. Ecol. Sociobiol.* **26**, 17–29 (1990).

Acknowledgements

We thank the Department of Forest Engineering at the Federal University of Viçosa for allowing us to perform this study at the Research Station of Mata do Pariso and Dr. Simon Elliot for hosting us in his laboratory. We are grateful to Charissa de Bekker who helped capture the behavior in Supplementary Video S9. This work was supported in part by National Science Foundation Grants IOS-1558062 and EEID 1414296 to D.P.H., NSF CCF-1617735 to D.Z.C. and NIH Grant R01 GM116927-02 to D.P.H. and D.Z.C.

Author Contributions

D.P.H., R.G.L. and N.I. conceived and designed the study. N.I. and C.K. performed the field work with technical input from R.G.L., Y.Z. and D.Z.C. created the computer model and processed the data. N.I. analyzed the data and wrote the manuscript with guidance from D.P.H. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-49655-3.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Appendix B

Supplementary Material for Chapter 3

1. Calculation of model time step

Our model time step is based on the average time it takes for a foraging *Camponotus rufipes* ant to move one body length. Standardizing movement data by the amount of time needed to move one body length prevents unrealistic turning values per move and allows better comparison of individuals (Tourtellot et al., 1991).

To determine the average body length of a foraging *Camponotus rufipes* ant, we measured ants from video frames in the videos analyzed in Imirzian et al. (2019). All videos can be accessed at https://doi.org/10.26207/q14b-gx36. Using ImageJ (Rueden et al., 2017), we measured the body length of each ant in pixels. Body length was measured from the tip of mandibles to the tip of the gaster (ant abdomen). We only measured ants that were in full view from overhead (Fig AB-1). We used each video's conversion factor (found in the Supp. Material of Imirzian et al., 2019) to convert the length in pixels to centimeters. We measured 15 ants from four different colonies for a total of 60 ants. Average body length was 0.81cm ± 0.12 cm (standard deviation). We divided the body length by the average speed of the ant trajectories (5.15 cm/s) to determine 0.16 as the average time it takes an ant to go the distance of one body length. We rounded up to use 0.2 seconds for the trajectory sampling factor (Appendix B.2) and time step in the model.

Figure AB-1. Demonstration of how ants measured from videos.

Circled ant has a line running across body length which was used to measure average body length.

References

Imirzian, N., Zhang, Y., Kurze, C., Loreto, R.G., Chen, D.Z., Hughes, D.P., 2019. Automated tracking and analysis of ant trajectories shows variation in forager exploration. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-49655-3

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18, 529. https://doi.org/10.1186/s12859-017-1934-z

Tourtellot, M.K., Collins, R.D., Bell, W.J., 1991. The problem of movelength and turn definition in analysis of orientation data. J. Theor. Biol. 150, 287–297. https://doi.org/10.1016/S0022-5193(05)80428-X

2. Calculation of ant movement parameters

To determine the movement parameters for this species of ant, we used the full tracking dataset from Imirzian et al. (2019) available at https://doi.org/10.26207/q14b-gx36. This dataset includes the trajectories for 64,383 *Camponotus rufipes* ants foraging on trails in the rainforest. Ant locations were recorded every video frame so trajectories are at a resolution of 25 or 30 location points per second, depending on the native frame rate of the camera. We first resampled our trajectories according to the model time step (see Appendix B.1 for calculation of the model time step). We used the R package 'trajr' (McLean and Volponi, 2018) to resample the trajectories every 0.2 seconds then calculated the turning angle and step length for each step of a trajectory. Most trajectories had an average *step-length* close to 1 (mean = 0.93; Figure AB-2) with a range from 0.01 to 2.61 cm.

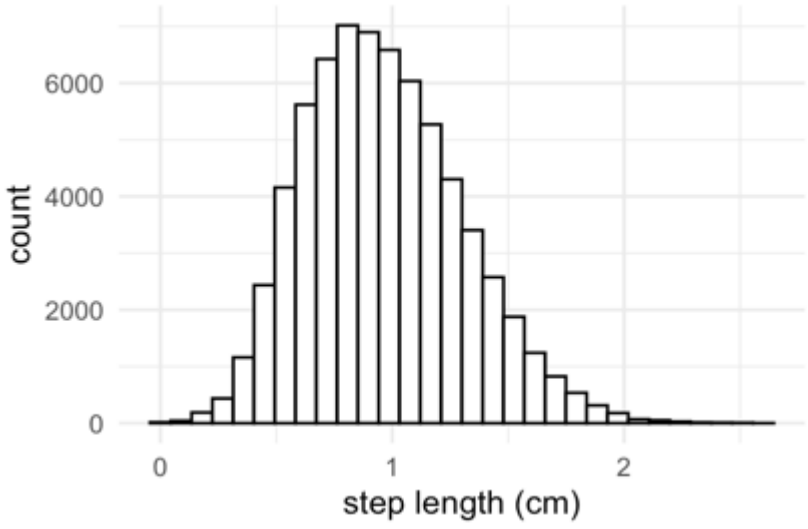


Figure AB-2. Distribution of mean length between steps for all ant trajectories.

We found the turning angle from the difference in angular orientation between adjacent time steps. The mean turning angles for all trajectories followed a normal distribution, with most trajectories averaging around a 0 degree turn (mean = -0.07; SD=8.67; Fig. AB-3A). The turning-index was calculated as the standard deviation of the turning angles. The mean turning-index for ant trajectories was 28.6 degrees (SD=16.89) and ranged from 0.65 to 92.2 (Fig. AB-3B).

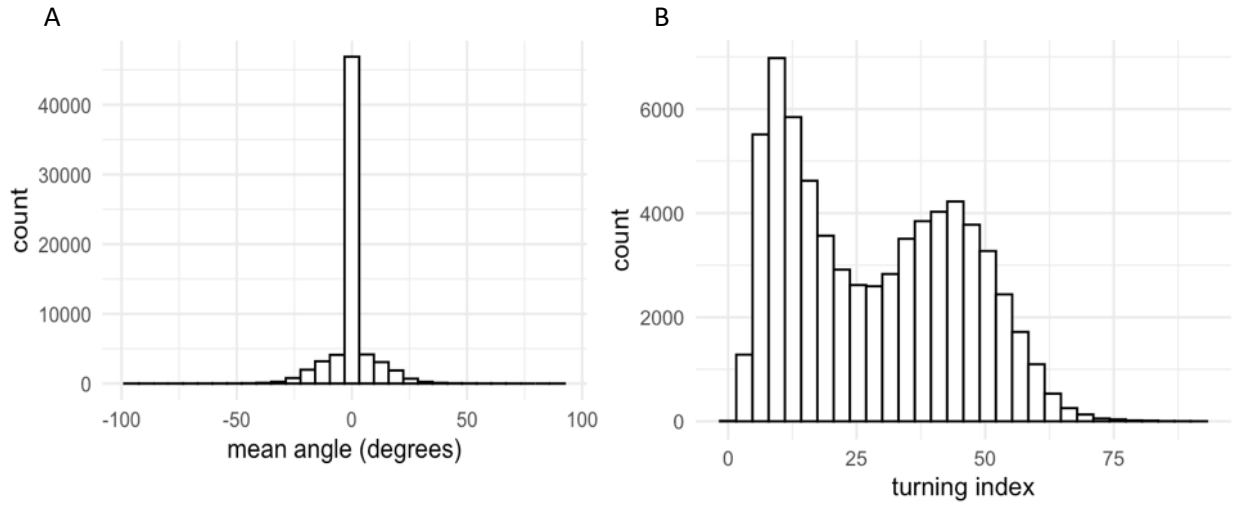


Figure AB-3. (A) Distribution of the mean turning angles for all field ant trajectories. (B) Distribution of the turning rates for all field ant trajectories.

References

Imirzian, N., Zhang, Y., Kurze, C., Loreto, R.G., Chen, D.Z., Hughes, D.P., 2019. Automated tracking and analysis of ant trajectories shows variation in forager exploration. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-49655-3

McLean, D.J., Volponi, M.A.S., 2018. trajr: An R package for characterisation of animal trajectories. Ethology 124, 440–448. https://doi.org/10.1111/eth.12739

3. Time-on-trail analysis

We looked at how specifying the time ants walk on the trail before they can move throughout the entire environment to end up on a cadaver location impacts the results. To test the likely range of time ants are spending on the trail, we assigned all ants in a model run to spend the same amount of time on the trail and investigated how altering *time-on-trail* impacted the proportion of ants on or near a target (measured by equation 1 (*f)* in the main text). We tested how the results change from changing *time-on-trail* along a range from 150 ticks (30 seconds) to 180,000 ticks (60 minutes). We tested this using two opposite movement styles at the high and low end of the parameter input distribution: fast moving ants (*step-length* = 2.5 and *turning-index* = 20) and slow-moving ants (*step-length* = 0.5 and *turning-index* = 70). All runs had a time limit of 108,000 ticks and we performed 10 runs per nest for each parameter combination. We found that for fast ants, as *time-on-trail* increased for a given parameter combination, the number of ants reaching a target decreased (Fig. AB-4). In contrast, the *time-on-trail* has little impact on the number of ants reaching a target for slow moving ants (Fig. AB-4). Thus, for our parameter searches, individual ants received a random value between 40 seconds and 10 minutes, as this would allow the ants to reach targets regardless of the movement parameters.

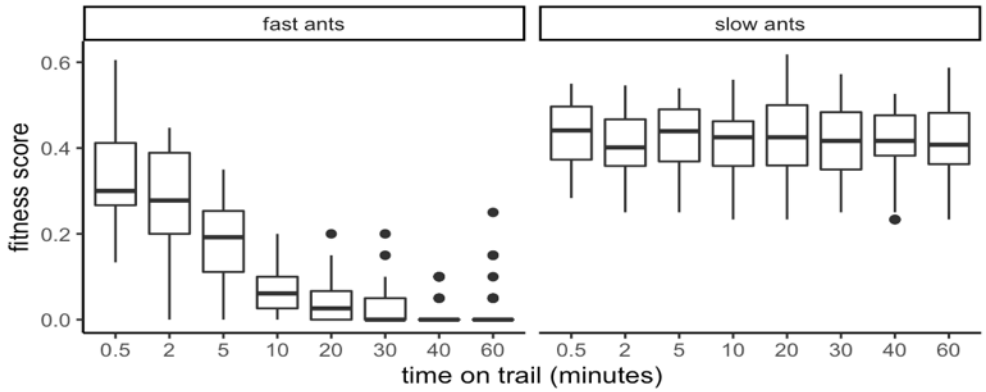


Figure AB-4. All ants in a model followed the trail for the time specified by *time-on-trail* and the number of ants reaching targets was measured by the fitness score (*f*).

4. Trail movement

We tested how specification of ant movement on trails impacts the results with three different setups (see section 7.3 in the chapter 3). We ran 10 parameter searchers for each setup and found they performed similarly as measured by the fitness equation, *f* (ANOVA; $F_{(1,118)} = 0.836$, $p = 0.36$; Table D.1). Interestingly, in setup 3, when turning rate on the trail was selected through parameter search, it resulted in a turning index close to that off of the trail: a mean of 73.48 on the trail compared to 70.85 off the trail (Table AB-1). Additionally, in setup 2, when ants had a low turning rate on the trail, the step size found from the parameter search was reduced, indicating slower movement is required for more ant agents to reach the targets when turning rate is increased. This result is replicated in the main analysis (Fig. 3-3).

Table AB-1. Comparison of results for three different ways of specifying movement on trails. The fitness equation specified by f can be found in the main text. 'Turning on trail' refers to how ants move when they are following the trail, while 'turning off trail' refers to how ants move once they have moved off of the trail.

Setup	Turning on trail	Turning off trail	*f* (max)	*f* (mean)	Step size (mean)	Turning index – on trail (mean)	Turning index – off trail (mean)
1	*turning-index*	*turning-index*	0.705	0.576	1.61	73.67	73.67
2	Mean from field data (low turning)	*turning-index*	0.704	0.566	1.12	32.00	60.53
3	*trail-turning-index*	*turning-index*	0.685	0.564	1.71	73.48	70.85

Appendix C

Supplementary Material for Chapter 4

Table AC-1. Results from Monte-Carlo simulations investigating whether the cadavers are located closer together than expected from complete spatial randomness.

The average distance to the nearest neighbour (ann) of a cadaver was first calculated for all trees with 15 or more cadavers. We then randomly distributed the same number of cadavers in same tree space and calculated the average nearest neighbour for the simulated distribution. We then counted the number of times the actual average nearest neighbour was greater than the simulated average nearest neighbour (ann) for 1000 simulations and divided that by the number of simulations (+ the actual observation) to get the p-value.

graveyard	tree	number of cadavers	actual ann (cm)	simulated ann (cm)	simulations with actual ann > simulated ann	number of simulations	*p*
1	1	175	3.52	6.26	0	1000	0.000999
2	1	15	17.82	18.89	351	1000	0.351648
3	1	21	7.84	14.53	0	1000	0.000999
4	1	92	8.08	10.14	1	1000	0.001998
4	11	48	5.34	10.02	0	1000	0.000999
4	20	87	5.81	8.49	0	1000	0.000999
5	1	54	10.14	14.39	0	1000	0.000999
5	3	73	2.64	7.88	0	1000	0.000999
6	1	15	12.30	11.44	702	1000	0.702298

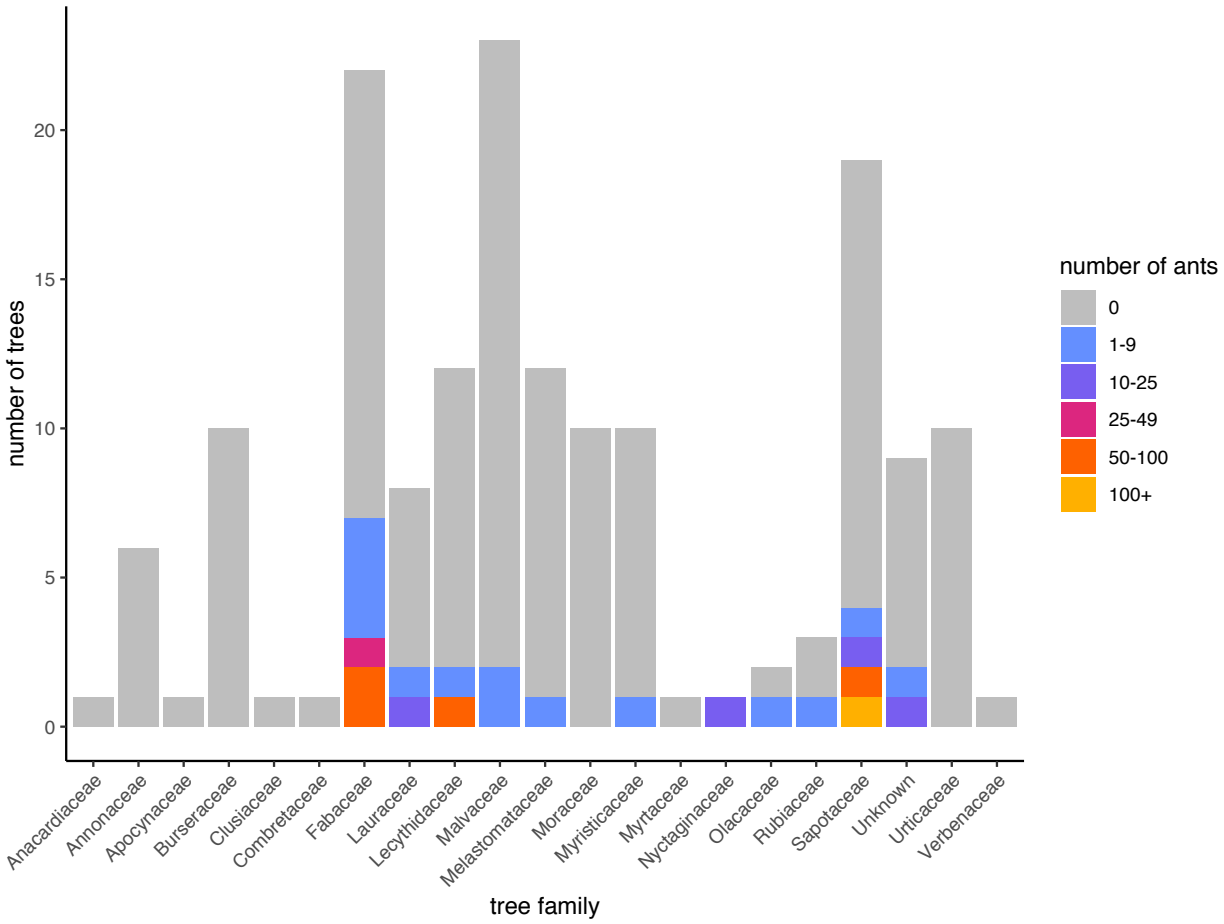


Figure AC-1. The total number of trees found in all 6 graveyards divided by tree family. The color of the bars indicates the number of trees found with the corresponding number of zombie ant cadavers.

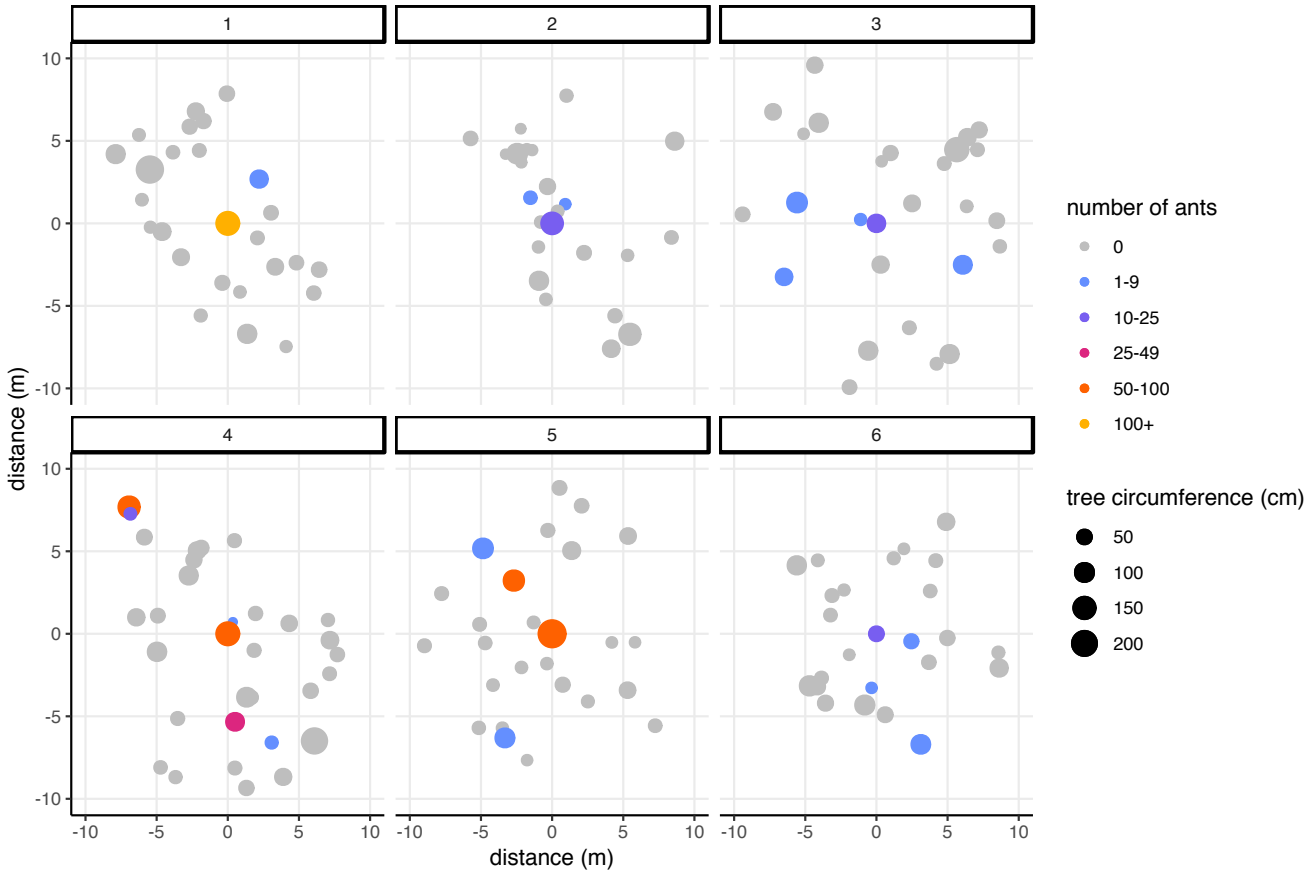


Figure AC-2. Distribution of trees in six different graveyards.

Size of dots reflect circumference of tree and colour of dots indicate number of *Ophiocordyceps kniphofiodes* infected *Cephalotes atratus* cadavers found on the tree. Overlapping of points are due to the minimizing of distribution into the available space.

Appendix D

Here today and there tomorrow: improving predictions of Desert Locust migration[4]

Abstract

The Desert Locust (*Schistocerca gregaria*) is one of the world's most destructive pest species due to its ability to switch from a solitary to a gregarious form. When gregarious, the insect aggregates in swarms covering up to 200 km^2 containing 40-80 million locusts per kilometer, each able to eat its own weight in food per day. The invasion area extends over 30 million km^2 of land from West Africa to western India, threatening the food of up to 20 million people. The expansive area covered by the Desert Locust is a huge barrier to control operations, as it is often unknown where a swarm will move next. However, today's technological capabilities should offer solutions to improve predictions of Desert Locust migration. In this review, I discuss what is known about the factors influencing where swarms of locusts fly. A tremendous amount of research has focused on this species, synthesizing this research with current technologies could gain an understanding into how to track and control this significant pest.

[4] Additional study completed during PhD

Introduction and basic biology

Within its solitary phase, the Desert Locust (*Schistocerca gregaria*) causes little harm and exists throughout its breeding range at low densities. However, if locust density at a location crosses above a threshold, the locusts undergo a phase transformation to a gregarious form, involving a full change in morphology, physiology, and behavior (Maeno & Tanaka, 2008; Pener & Yerushalmi, 1998; Simpson et al., 2005; Sword et al., 2000). Despite the apparent complexity, phase transformation is simply stimulated by increased contact to the insect's hind legs (Simpson et al., 2001). Once in the gregarious form, the locust is extremely destructive, forming swarms with densities of 50 million locusts/ km^2 and moving over 100 kilometers in a day (Kennedy, 1951; Rainey, 1963).

When the desert locust is primarily solitarious throughout its range, this period of time is known as a recession. As the locust begins to multiply and increase in density, leading to gregarization and swarm formation, an outbreak can occur. If outbreaks are left uncontrolled, they can lead to upsurges, and eventually plagues, which are periods of time of widespread and heavy infestations (Hemming et al., 1979; Roffey et al., 1970). In the 20^{th} century, there were 7 major plagues, leading to a significant amount of damage to crops (Magor et al., 2008). Consequently, there is substantial interest in understanding locust biology and movement ecology to better understand how to control outbreaks.

Development of the desert locust takes place in three stages, through egg, nymph, and adult forms. Eggs are typically laid 5-10 cm deep in sandy soil, and there must be moisture in the soil for the female to lay her eggs (Hunter-Jones, 1964). Eggs can take anywhere from 10-65 days to develop depending on environmental conditions, with development time shortening at higher soil temperatures (Hunter-Jones, 1970; Nishide et al., 2015). Females usually lay around 80 eggs

per pod in their gregarious form, and between 90 - 160 in their solitary form (Ashall & Ellis, 1962; Roffey & Popov, 1968). Typically, eggs laid by gregarious locusts show synchronicity, hatching within a few hours of dawn (Ashall & Ellis, 1962; Ellis & Ashall, 1957; Padgham, 1981; Wardhaugh et al., 1969).

Once an egg hatches, it passes through 5 nymphal stages before molting into an immature adult form, although some small solitary nymphs will pass through 6 stages (Maeno & Tanaka, 2008). The nymphs are known as 'hoppers' due to their tendency to hop from place to place. Hoppers can eat their own weight in vegetation in each day (Davey, 1954). In the solitary form, hoppers behave independently while roosting and feeding (Maxwell-Darling, 1934; Roffey & Popov, 1968). However, in the gregarious state, their daily behavior follows a coordinated pattern where the insects form bands that march in a uniform fashion. At night and mid-day when the temperature is highest, the locust nymphs can be found roosting in vegetation. In the morning and mid-afternoon, the locusts spend 2-3 hours marching, traveling up to 30 kilometers (Ellis & Ashall, 1957; Kennedy, 1939; Maxwell-Darling, 1936; Pedgley, 1981).

Like egg development, hopper development is dependent on temperature, as well as food availability, population density, and humidity (Hamilton, 1936; Maeno & Tanaka, 2008; Wardhaugh et al., 1969). After its last instar, the hopper molts into a fledgling with wings not fully developed making it unable to migrate. After spending approximately 10 days as a fledgling, the locusts take flight.

Locust migration

Similar to hopper bands, gregarious adult locusts show a predictable daily pattern in activity. They roost in vegetation overnight, then take off in the morning once they are sufficiently warm (Gunn et al., 1945; Waloff, 1946b). Departure takes place in a series of steps,

first with small numbers of locusts flying in many directions, then more locusts joining in with increasingly coordinated directions, until mass departure with the entire swarm rising to fly in one direction (Kennedy, 1951). The locust swarm will fly for 9-10 hours before settling in the evening before sunset, although heavy rain or cloud cover may inhibit flying and cause them to settle (Gunn et al., 1945; Waloff & Rainey, 1951). Swarms take on various formations in the air, sometimes flying in a low flying horizontal sheet known as a stratiform formation, or extending vertically as a cumuliform shape (Rainey, 1958).

General patterns in where locusts breed and move are determined by wind patterns, precipitation, and vegetation availability. Coastal areas near the Red Sea and Gulf of Aden serve as a reservoir for breeding during recessions, due to consistent rainfall, as well as some areas in northwestern Africa (Skaf et al., 1990). These areas, along with parts of Iran, Pakistan, and central Saudi Arabia, compose the winter and spring breeding areas (Symmons & Cressman, 2001). In the summer, locusts migrate to other breeding areas: from the Gulf of Aden and the Red Sea to other parts of eastern Africa (Sudan, Eritrea, Ethiopia), from Iran and Pakistan to the Indo-Pakistan border, and from northwest Africa into Sahel region of West Africa.

Importantly, flight behavior differs between swarming and solitary locusts. Swarms migrate almost exclusively by day, except for some low-density and immature swarms (Roffey, 1963), while non-swarming populations tend to fly at night (Roffey & Popov, 1968). The gregariousness of swarms allows them to enter territories unusual for recession populations, expanding the invasion area by almost double the size of the recession area. Additionally, a variety of long-distance migrations have been documented by swarms, from Morocco to Portugal (Waloff, 1946a) and even from western Africa to South America (Rosenberg & Burt, 1999), a testament to the enhanced flying ability of locusts in swarms (Lorenz, 2009).

The precise location of locust migration can be highly variable. The difficulty in knowing exactly where locusts will show up next can inhibit control operations during outbreaks. Current

work by the Hughes Lab through PlantVillage has created the eLocust3m app to enable more accurate collection of data on locust swarms. This has resulted in over 16,000 new records of locusts collected by the app and mapped with historic examples (1985-2019) on this ArcGIS map https://arcg.is/0aHGHi. To support this work, it is important to examine the factors that influence swarm movement. One important factor is wind. The ArcGIS board already integrates wind dispersion models by NOAA which have been used by UN FAO to model movement. But these are unparametrized models and it is likely that the literature contains important information useful to understanding the role wind plays in predicting locust movements. Here I set out to review the literature to contribute to the ongoing work of PlantVillage with the UN FAO to monitor locust swarms.

Swarm movement

I systematically searched all research articles from 1930 - present for information on the movement of desert locust swarms relative to wind. I found a total of 12 field studies across the invasion region that connected swarm movements to wind patterns (Table D-1). Interestingly, no recent studies have looked at wind patterns relative to swarm movement, with 8 out of 12 studies taking place between 1940-1951 by a total of 4 researchers.

Generally, swarms move downwind (Draper, 1980; Kennedy, 1951; Rainey, 1963; Roffey, 1963; Waloff, 1972). However, at low wind speeds (<4 m/s), when locust flight speed is greater than wind speed, locusts may fly upwind or crosswind (Kennedy, 1951; Waloff & Rainey, 1951). Convection currents alter the structure of locust swarms, as cumuliform swarms are associated with strong up-currents, while lower-flying stratiform swarms are more likely in the absence of vertical air movements (Rainey & Waloff, 1951). In a cumuliform swarm, where locusts are spread out vertically, it is unclear at what height the wind has the largest impact on

swarm movement. The flying height of locust swarms ranges from 15-1700 meters above the ground, with cumuliform swarms generally higher above the ground (Pedgley, 1981).

Although the evidence indicates that locust swarms consistently move downwind, swarms may not necessarily migrate with prevailing winds. Swarms may stay in an area until wind patterns reverse, such as when swarms migrate in the rare cross-sea winds from Arabia to central Sudan or northwards across the Sahara desert (Symmons & Cressman, 2001). Consequently, it is important to understand the factors influencing swarm takeoff and settling (Table D-2). Temperature plays a large role in initiating swarm departure, as the locust's flight muscles must be warm enough to fly (Gunn et al., 1945; Waloff & Rainey, 1951). Conversely, if the temperature is too hot, often during midday, locusts may settle to cool down (Pedgley, 1981). Overall, studies show that the time swarms takeoff can be highly variable, ranging from 2-6 hours after sunrise, depending on the temperature. Gunn et al. (1945) and Waloff & Rainey (1951) developed equations for estimating the temperature of swarm departure based on the previous day's temperature, but the accuracy varies (see page 56 in Pedgley, 1981). A better understanding of the factors controlling mass departure of swarms would help immensely with predicting swarm movement, as it would allow researchers to use the precise wind conditions at the time of takeoff.

As far as how laboratory studies contribute to our understanding, a few have looked at the mechanics of tethered locust flight (Mappes & Homberg, 2004; Preiss & Gewecke, 1991; Preiss & Spork, 1993; Taylor & Thomas, 2003). Some laboratory studies have indicated locusts respond to polarized light and could use this for orientation (Bech et al., 2014; Beetz et al., 2016; Mappes & Homberg, 2004; Shashar et al., 2005), although this has not been thoroughly investigated in the field, apart from a few observations by Kennedy (1951). Observations of a locust swarm avoiding flying over a body of water (the Gulf of Aqaba in 2004) have indicated that locusts use light polarization in swarm migration (Shashar et al., 2005). A recent study further demonstrated the locust's ability to use solar polarization patterns (Zittrell et al., 2020), promoting the idea of an

internal sun compass (reviewed in Homberg, 2015). More work is needed to elucidate how this factor interacts with wind in determining swarm direction.

Possible research directions

Digitizing records

Many older studies on desert locusts collected detailed data on the location of swarms over time. If digitized, this data could be correlated with historical climate conditions and used in machine learning models to form a prediction of where locusts move over time. A few suggestions of good sources containing this data include:

- Kennedy (1951), Table 2, page 168: list of observations of swarms giving location, date, wind condition, swarm direction and characteristics from 1942-1944
- Waloff (1946a), Appendix 3, pages 73-74: chart showing number of swarms recorded at different locations for 1928-31 and 1941, 1944
- Rainey & Waloff (1948), all figures: trajectories of swarm movements and wind direction in the Gulf of Aden area from 1943-1946
- Rainey (1963), Figure 2, 8-16, Table 2: trajecoteries of swarm movements
- Pedgley (1980): 24 maps accompanying manual (Pedgley, 1981) showing frequency of locust observations across region over 37-year period 1939-1975

Tracking locusts

Technology has advanced to the point where individual tracking devices are small enough to be placed on insects (Kissling et al., 2014). Tracking devices have been used on at least 94 different arthropod species (Batsleer et al., 2020), including on Orthopteran species (Fornoff et al., 2012; Gwynne & Kelly, 2018; Lorch et al., 2005; Srygley et al., 2009; Watts et al., 2011). Desert locusts offer challenges for this technique due to height and distance flown, as well as the remoteness of some of the breeding sites. However, if radio tracking could be adapted for desert locusts, this would be immensely helpful for learning the precise trajectories of swarms, which could then be used to improve predictive models.

Sun compass experiments

Since Kennedy (1951), there have been no field studies investigating how solar light impacts locust flight. Given the recent laboratory research indicating light as a possible factor in locust navigation, it would be extremely useful to replicate and expand on this in a natural setting. Moreover, a laboratory study singling out each navigation factor, limiting the locust's sensory capabilities one-by-one, would help elucidate what plays the strongest role in influencing locust flight direction. A huge barrier to laboratory investigations is how behavior differs when locusts are a part of a swarm, which is difficult to replicate in controlled conditions. Thus, a mixture of these different approaches would be best.

Conclusions

To improve control operations, we need a better understanding of the relative role of each factor influencing locust swarm movement. After many foundational studies on swarm movement in the mid 20th century, locust research has taken a different direction with fewer field studies investigating locust movement. Yet, swarm migration is still not fully understood, and more research is needed to understand this complex phenomenon. To improve our predictions of desert locust movement, I suggest digitizing earlier records to improve predictive models, remote tracking of swarms, and controlled studies isolating the factors involved in locust migration. Additionally, engaging the populations most affected by outbreaks in research is the best way to ensure studies are happening in high impact areas. This work is crucial to protect the food of millions of people.

Tables

Table D-1. Studies investigating locust movement relative to wind direction, in order of year.

Study Type	Location	Main Observations	Reference
Field	Egypt	Flight direction corresponds with prevailing winds	(Ballard et al., 1932)
Field	India	Eastward and northward migration in spring and summer coincides with south-westerly winds; swarms in central Indo-Gangetic plain swept east by westerly winds in September; swarms in western Rajputana swept south westwards to Baluchistan	(Rao, 1942)
Field	Morocco to Portugal	Direction of long-distance migration of swarm corresponded with direction of air movement	(Waloff, 1946a)
Field	East Africa	Overall patterns show swarm migrations follow prevailing wind patterns (downwind). However, swarm movements in northwestern Kenya, Ethiopia and part of the Somali Peninsula were observed against the prevailing winds in October to March (but mentioned it may be due to lack of data on local wind). Found older swarms will move against prevailing wind even when younger swarms did not.	(Waloff, 1946b)
Field	Gulf of Aden	Convection currents affect flying - frequent association of swarm movements with rising sand, dust storms	(Rainey & Waloff, 1948)
Field	Entire Region	Locusts change direction to downwind when wind speed increases to greater than 4 m/s	(Kennedy, 1951)
Field	East Africa	At wind speeds less than flying speed of locusts, takeoff into the wind. Locusts at winds above flying speed altered course to down-wind.	(Waloff & Rainey, 1951)
Field	East Africa	Cumuliform swarms associated with convection currents	(Rainey & Waloff, 1951)
Field	Entire Region	Major swarm displacements downwind towards areas of low-level wind convergence; association between the Intertropical Convergence Zone and movement of locust swarms	(Rainey, 1951)
Field	Kenya	Hourly movement of swarms followed the direction of wind. Areas with uniform wind patterns tended to show systematic displacements downwind while complex wind-fields led to complex tracks where swarms would cross over trajectories or show little overall displacement.	(Rainey, 1963)

Field	Entire Region	Orientation of swarm mostly with wind. Lower flying locusts will fly into wind but this is reduced at high winds. Downwind displacements likely mostly with denser and high-rising swarms	(Waloff, 1972)
Field	Eastern Africa	No evidence that swarms follow a preferred compass direction, rain-in-sight, or orient towards high ground. Swarms show significant deviation from downwind direction, but consistent with errors of calculating mean wind over range of swarm height	(Draper, 1980)

Table D-2. Other factors that impact Desert Locust swarm movement.

	Departure from roosting site	**Swarm Displacement**	**Settling from flight**
Factor	Effect	Effect	Effect
Wind	Drop in wind speed leads to outburst of flying (Kennedy, 1951) High wind (6-10 m/s) may hinder flight, swarms shown to only take to the air when wind calms even with high air temperature and sunshine (Waloff, 1972)	Generally swarms move downwind (Table D-1) At low wind speeds (< 4m/s) can move against wind (J. S. Kennedy, 1951)	Wind gusts can lead to settling of some locusts (Kennedy, 1951; Waloff, 1972) (Kennedy 1951; Waloff 1972)
Temperature	Take off at: Sunny: >15-17 C Cloudy: >23-24 C (immature); >26 C (mature) (Gunn et al., 1945; Waloff & Rainey, 1951) Air temperature of the previous day correlated with air temperature at mass departure (Gunn et al., 1945; Waloff & Rainey, 1951) T=5.6+0.74 X, where X is mean temp of previous day, and T is the temp at mass departure (Waloff & Rainey, 1951); however, equation is not very reliable (D. E. Pedgley, 1981)	Limited displacement at low temperatures as few locusts can fly (Rainey, 1963)	Swarms settled when clouds pass over sun at air temp <23 C, but continued flying at higher temps (Gunn et al., 1945) May also settle midday at high temperatures (D. E. Pedgley, 1981)
Other locusts	Mass departure when basking locusts join swarm flying overhead (Kennedy 1951; Gunn et al. 1945)	Two meeting swarms will change orientation to denser swarm to be in alignment (Kennedy, 1951) Larger, high-flying swarms move faster than smaller, low-flying	

		swarms (Pedgley, 1981)	
Vegetation	Swarms will leave location when not enough vegetation to continue feeding		Fledglings in Iran showed some attraction to large trees or tallest vegetation available, took short flights and collected in large numbers in oases (Kennedy, 1951)
Precipitation	Dry weather = swarms more likely to leave an area (Waloff, 1946b) Fewer flying swarms during rainy/breeding season (Waloff, 1946b)		Swarms will settle in heavy rain (Waloff & Rainey, 1951) End migration when reach area with sufficient moisture for laying
Solar Orientation	Cloud cover may delay the initiation of flying	Dorsal rim area has specialized photoreceptors with receptive fields that can perceive nearly the entire sky (Bech et al., 2014; Schmeling et al., 2015) Laboratory studies provide some evidence of orientation using a sun compass (reviewed in Homberg, 2015), but yet to be shown in field	Passing of cloud over sun causes settling (Gunn et al., 1945) Swarms settle from 2 hours before sunset to ½ an hour after sunset (Gunn et al., 1945; Waloff & Rainey, 1951)
Locust Age	Locusts will begin migration once their wings have hardened sufficiently for long-distance flight Immature swarms may be more likely to fly during the nighttime	Older/mature locusts better able to fly against wind compared to immature locusts. Fledglings only take short low flights for several days after fledging (Kennedy, 1951)	

References

Ashall, C., & Ellis, P. E. (1962). Studies on numbers and mortality in field populations of the desert locust (Schistocerca gregaria Forskål). *Anti-Locust Bulletin*, *38*, 1–59.

Ballard, E., Mistikawi, A. M., & El Zoheiry, M. S. (1932). The Desert Locust, Schistocerca gregaria Forsk., in Egypt. *The Desert Locust, Schistocerca gregaria Forsk., in Egypt.*, *110*. http://www.cabdirect.org/cabdirect/abstract/19330501037

Batsleer, F., Bonte, D., Dekeukeleire, D., Goossens, S., Poelmans, W., Cruyssen, E. V. der, Maes, D., & Vandegehuchte, M. L. (2020). The neglected impact of tracking devices on terrestrial arthropods. *Methods in Ecology and Evolution*, *11*(3), 350–361. https://doi.org/10.1111/2041-210X.13356

Bech, M., Homberg, U., & Pfeiffer, K. (2014). Receptive Fields of Locust Brain Neurons Are Matched to Polarization Patterns of the Sky. *Current Biology*, *24*(18), 2124–2129. https://doi.org/10.1016/j.cub.2014.07.045

Beetz, J. M., Pfeiffer, K., & Homberg, U. (2016). Neurons in the brain of the desert locust Schistocerca gregaria sensitive to polarized light at low stimulus elevations. *Journal of Comparative Physiology A*, *202*(11), 759–781. https://doi.org/10.1007/s00359-016-1116-x

Davey, P. M. (1954). Quantities of Food eaten by the Desert Locust, Schistocerca gregaria (Forsk.), in Relation to Growth. *Bulletin of Entomological Research*, *45*(3), 539–551. https://doi.org/10.1017/S0007485300029618

Draper, J. (1980). The Direction of Desert Locust Migration. *Journal of Animal Ecology*, *49*(3), 959–974. JSTOR. https://doi.org/10.2307/4238

Ellis, P. E., & Ashall, C. (1957). Field Studies on diurnal Behaviour, Movement and Aggregation in the Desert Locust (Schistocerca gregaria Forskål). *Anti-Locust Bulletin*, *25*.

Fornoff, F., Dechmann, D., & Wikelski, M. (2012). Observation of movement and activity via radio-telemetry reveals diurnal behavior of the neotropical katydid Philophyllia Ingens (Orthoptera: Tettigoniidae). *Ecotropica*, *18*(1), 27–34.

Gunn, D. L., Perry, F. C., Seymour, W. G., Telford, T. M., Wright, E. N., & Yeo, D. (1945). Mass Departure of Locust Swarms in Relation to Temperature. *Nature*, *156*(3969), 628–629. https://doi.org/10.1038/156628a0

Gwynne, D. T., & Kelly, C. D. (2018). Successful use of radiotransmitters in tracking male tree wētā Hemideina crassidens (Orthoptera: Tettigonioidea: Anostostomatidae). *New Zealand Entomologist*, *41*(1), 25–28. https://doi.org/10.1080/00779962.2018.1501138

Hamilton, A. G. (1936). The Relation of Humidity and Temperature to the Development of Three Species of African Locusts—Locusta Migratoria Migratorioides (r. & F.), Schistocerca Gregaria (forsk.), Nomadacris Septemfasciata (serv.). *Transactions of the Royal Entomological Society of London*, *85*(1), 1–60. https://doi.org/10.1111/j.1365-2311.1936.tb00231.x

Hemming, C. F., Popov, G. B., Roffey, J., Waloff, Z., Gunn, D. L., & Rainey, R. C. (1979). Characteristics of Desert Locust plague upsurges. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *287*(1022), 375–386. https://doi.org/10.1098/rstb.1979.0069

Homberg, U. (2015). Sky Compass Orientation in Desert Locusts—Evidence from Field and Laboratory Studies. *Frontiers in Behavioral Neuroscience*, *9*. https://doi.org/10.3389/fnbeh.2015.00346

Hunter-Jones, P. (1964). Egg development in the Desert Locust (Schistocerca gregaria Forsk.) in relation to the availability of water. *Proceedings of the Royal Entomological Society of London. Series A, General Entomology*, *39*(1–3), 25–33. https://doi.org/10.1111/j.1365-3032.1964.tb00781.x

Hunter-Jones, P. (1970). The effect of constant temperature on egg development in the desert locust Schistocerca gregaria (Forsk.). *Bulletin of Entomological Research*, *59*(4), 707–718. https://doi.org/10.1017/S0007485300003722

Kennedy, J. S. (1939). The Behaviour of the Desert Locust (schistocerca Gregaria (forsk.)) (orthopt.) in an Outbreak Centre. *Transactions of the Royal Entomological Society of London*, *89*(10), 385–542. https://doi.org/10.1111/j.1365-2311.1939.tb00735.x

Kennedy, J. S. (1951). The Migration of the Desert Locust (Schistocerca gregaria Forsk.). I. The Behaviour of Swarms. II. A Theory of Long-Range Migrations. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *235*(625), 163–290. JSTOR.

Kissling, W. D., Pattemore, D. E., & Hagen, M. (2014). Challenges and prospects in the telemetry of insects. *Biological Reviews*, *89*(3), 511–530. https://doi.org/10.1111/brv.12065

Lorch, P. D., Sword, G. A., Gwynne, D. T., & Anderson, G. L. (2005). Radiotelemetry reveals differences in individual movement patterns between outbreak and non-outbreak Mormon cricket populations. *Ecological Entomology*, *30*(5), 548–555. https://doi.org/10.1111/j.0307-6946.2005.00725.x

Lorenz, M. W. (2009). Migration and trans-Atlantic flight of locusts. *Quaternary International*, *196*(1), 4–12. https://doi.org/10.1016/j.quaint.2007.09.038

Maeno, K., & Tanaka, S. (2008). Phase-specific developmental and reproductive strategies in the desert locust. *Bulletin of Entomological Research*, *98*(5), 527–534. https://doi.org/10.1017/S0007485308006044

Magor, J. I., Lecoq, M., & Hunter, D. M. (2008). Preventive control and Desert Locust plagues. *Crop Protection*, *27*(12), 1527–1533. https://doi.org/10.1016/j.cropro.2008.08.006

Mappes, M., & Homberg, U. (2004). Behavioral analysis of polarization vision in tethered flying locusts. *Journal of Comparative Physiology: Neuroethology, Sensory, Neural, and*

Behavioral Physiology, A; Heidelberg, *190*(1), 61–68. http://dx.doi.org.ezaccess.libraries.psu.edu/10.1007/s00359-003-0473-4

Maxwell-Darling, R. C. (1934). The Solitary Phase of Schistocerca Gregaria, Forsk., in North-Eastern Kordofan (Anglo-Egyptian Sudan). *Bulletin of Entomological Research*, *25*(1), 63–83. https://doi.org/10.1017/S0007485300012517

Maxwell-Darling, R. C. (1936). The Outbreak Centres of Schistocerca gregaria, Forsk., on the Red Sea Coast of the Sudan. *Bulletin of Entomological Research*, *27*(1), 37–66. https://doi.org/10.1017/S0007485300058107

Nishide, Y., Tanaka, S., & Saeki, S. (2015). Egg hatching of two locusts, Schistocerca gregaria and Locusta migratoria, in response to light and temperature cycles. *Journal of Insect Physiology*, *76*, 24–29. https://doi.org/10.1016/j.jinsphys.2015.03.010

Padgham, D. E. (1981). Hatching rhythms in the desert locust, Schistocerca gregaria. *Physiological Entomology*, *6*(2), 191–198. https://doi.org/10.1111/j.1365-3032.1981.tb00641.x

Pedgley, D. E. (1980). *Desert locust forecasting manual (Volume 2 of 2)*. Centre for Overseas Pest Research.

Pedgley, D. E. (1981). *Desert locust forecasting manual (Volume 2 of 2)*. Centre for Overseas Pest Research. https://gala.gre.ac.uk/id/eprint/11863/

Pener, M. P., & Yerushalmi, Y. (1998). The physiology of locust phase polymorphism: An update. *Journal of Insect Physiology*, *44*(5), 365–377. https://doi.org/10.1016/S0022-1910(97)00169-8

Preiss, R., & Gewecke, M. (1991). Compensation of Visually Simulated Wind Drift in the Swarming Flight of the Desert Locust (schistocerca Gregaria). *Journal of Experimental Biology*, *157*(1), 461–481.

Preiss, R., & Spork, P. (1993). Flight-phase and visual-field related optomotor yaw responses in gregarious desert locusts during tethered flight. *Journal of Comparative Physiology A*, *172*(6), 733–740. https://doi.org/10.1007/BF00195398

Rainey, R. C. (1951). Weather and the Movements of Locust Swarms: A New Hypothesis. *Nature*, *168*, 1057–1060. https://doi.org/10.1038/1681057a0

Rainey, R. C. (1958). Some observations on flying locusts and atmospheric turbulence in eastern Africa. *Quarterly Journal of the Royal Meteorological Society*, *84*(362), 334–354. https://doi.org/10.1002/qj.49708436204

Rainey, R. C. (1963). Meteorology and the migration of desert locusts. *World Meteorological Organization*, *138*.

Rainey, R. C., & Waloff, Z. (1948). Desert Locust Migrations and Synoptic Meteorology in the Gulf of Aden Area. *Journal of Animal Ecology*, *17*(2), 101–112. JSTOR. https://doi.org/10.2307/1472

Rainey, R. C., & Waloff, Z. (1951). Flying Locusts and Convection Currents. *Anti-Locust Bull.*, *9*, 51–70.

Rao, R. B. Y. R. (1942). Some Results of Studies on the Desert Locust (Schistocerca gregaria, Forsk.) in India. *Bulletin of Entomological Research*, *33*(4), 241–265. https://doi.org/10.1017/S0007485300026572

Roffey, J. (1963). Observations on gliding in the desert locust. *Animal Behaviour*, *11*(2), 359–366. https://doi.org/10.1016/S0003-3472(63)80126-8

Roffey, J., & Popov, G. B. (1968). Environmental and behavioural processes in a desert locust outbreak. *Nature*, *219*(5153), 446–450.

Roffey, J., Popov, G., & Hemming, C. F. (1970). Outbreaks and recession populations of the desert locust Schistocerca gregaria (Forsk.). *Bulletin of Entomological Research*, *59*(4), 675–680. https://doi.org/10.1017/S0007485300003679

Rosenberg, J., & Burt, P. J. A. (1999). Windborne displacements of Desert Locusts from Africa to the Caribbean and South America. *Aerobiologia*, *15*(3), 167–175. https://doi.org/10.1023/A:1007529617032

Schmeling, F., Tegtmeier, J., Kinoshita, M., & Homberg, U. (2015). Photoreceptor projections and receptive fields in the dorsal rim area and main retina of the locust eye. *Journal of Comparative Physiology A*, *201*(5), 427–440. https://doi.org/10.1007/s00359-015-0990-y

Shashar, N., Sabbah, S., & Aharoni, N. (2005). Migrating locusts can detect polarized reflections to avoid flying over the sea. *Biology Letters*, *1*(4), 472–475. https://doi.org/10.1098/rsbl.2005.0334

Simpson, S. J., Despland, E., Hägele, B. F., & Dodgson, T. (2001). Gregarious behavior in desert locusts is evoked by touching their back legs. *Proceedings of the National Academy of Sciences*, *98*(7), 3895–3897. https://doi.org/10.1073/pnas.071527998

Simpson, S. J., Sword, G. A., & De Loof, A. (2005). Advances, Controversies and Consensus in Locust Phase Polyphenism Research. *Journal of Orthoptera Research*, *14*(2), 213–222. JSTOR.

Skaf, R., Popov, G. B., Roffey, J., Scorer, R. S., Hewitt, J., Rainey, R. C., Browning, K. A., Cheke, R. A., & Haggis, M. J. (1990). The Desert Locust: An international challenge. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *328*(1251), 525–538. https://doi.org/10.1098/rstb.1990.0125

Srygley, R. B., Lorch, P. D., Simpson, S. J., & Sword, G. A. (2009). Immediate protein dietary effects on movement and the generalised immunocompetence of migrating Mormon crickets Anabrus simplex (Orthoptera: Tettigoniidae). *Ecological Entomology*, *34*(5), 663–668. https://doi.org/10.1111/j.1365-2311.2009.01117.x

Sword, G. A., Simpson, S. J., El Hadi, O. T. M., & Wilps, H. (2000). Density–dependent aposematism in the desert locust. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1438), 63–68. https://doi.org/10.1098/rspb.2000.0967

Symmons, P., & Cressman, K. (2001). Desert locust guidelines: Biology and behaviour. *FAO, Rome*.

Taylor, G. K., & Thomas, A. L. R. (2003). Dynamic flight stability in the desert locust Schistocerca gregaria. *Journal of Experimental Biology*, *206*(16), 2803–2829. https://doi.org/10.1242/jeb.00501

Waloff, Z. (1946a). A Long-Range Migration of the Desert Locust from Southern Morocco to Portugal, with an Analysis of Concurrent Weather. Conditions. *Proceedings of the Royal Entomological Society of London. Series A, General Entomology*, *21*(10–12), 81–84. https://doi.org/10.1111/j.1365-3032.1946.tb01078.x

Waloff, Z. (1946b). Seasonal Breeding and Migrations of the Desert Locust (Schistocerca Gregaria Forskal) in Eastern Africa. *Anti-Locust Mem.*, *1*.

Waloff, Z. (1972). Orientation of flying locusts, Schistocerca gregaria (Forsk.), in migrating swarms. *Bulletin of Entomological Research*, *62*(1), 1–72. https://doi.org/10.1017/S0007485300003771

Waloff, Z., & Rainey, R. C. (1951). Field Studies on Factors affecting the Displacements of Desert Locust Swarms in eastern Africa. *Anti-Locust Bulletin*, *9*, 1–50.

Wardhaugh, K., Ashour, Y., Ibrahim, A. O., Khan, A. M., & Bassonbol, M. (1969). Experiments on the incubation and hopper development periods of the desert locust (Schistocerca gregaria Forskál) in Saudi Arabia. *Anti-Locust Bulletin*, *45*, 1–38.

Watts, C., Stringer, I., Thornburrow, D., & MacKenzie, D. (2011). Are footprint tracking tunnels suitable for monitoring giant weta (Orthoptera: Anostostomatidae)? Abundance,

distribution and movement in relation to tracking rates. *Journal of Insect Conservation*, *15*(3), 433–443. https://doi.org/10.1007/s10841-010-9321-3

Zittrell, F., Pfeiffer, K., & Homberg, U. (2020). Matched-filter coding of sky polarization results in an internal sun compass in the brain of the desert locust. *Proceedings of the National Academy of Sciences*, *117*(41), 25810–25817. https://doi.org/10.1073/pnas.2005192117

VITA

Natalie Imirzian

EDUCATION

The Pennsylvania State University, *University Park, PA*
Ph.D. in Entomology, anticipated May 2021
Graduate Certificate: Schreyer Institute Teaching

University of Michigan, *Ann Arbor, MI*
B.S. in Biology (Honors) and B.S. in Evolutionary Anthropology, May 2016
Graduated with High Distinction, *Phi Beta Kappa*

PUBLICATIONS

Imirzian, N., Araújo, J.P. and Hughes, D.P., 2020. A new zombie ant behavior unraveled: Aggregating on tree trunks. *Journal of Invertebrate Pathology*, *177*, p.107499.

Imirzian, N., Zhang, Y., Kurze, C., Loreto, R.G., Chen, D.Z. and Hughes, D.P., 2019. Automated tracking and analysis of ant trajectories shows variation in forager exploration. *Scientific Reports*, *9*(1), pp.1-10.

Hu, T., Zheng, H., Liang, C., Sirou, Z., **Imirzian, N.**, Zhang, Y., Wang, C., Chen, D.Z., and Hughes, D.P., 2020. AntVis: A Web-Based Visual Analytics Tool for Exploring Ant Movement Data. *Visual Informatics, 4*(1)*:* pp.58-70

HONORS AND AWARDS

2019	William Yendol Memorial Research Award, PSU
2018	Sigma-Xi Grant in Aid of Research, Sigma Xi: The Scientific Research Honor Society
2018	Runner-Up Life Science Symposium Student Competition, PSU
2018	College of Agricultural Sciences Graduate Student Competitive Grant, PSU
2018	Global Programs Travel Award, PSU
2018	Sahakian Travel Award, PSU
2016 - 2017	University Graduate Fellowship, PSU

TEACHING EXPERIENCE

Spring 2020	Guest Lecturer – Sociobiology, PSU
Fall 2017	Teaching Assistant – Sociobiology, PSU
Spring 2017	Teaching Assistant – The Insect Connection, PSU