Trail pheromone ecology of the pavement ant, *Tetramorium immigrans,* and the European fire ant, *Myrmica rubra*

by

Jaime M. Chalissery

B.Sc. (Biology), Simon Fraser University, 2018

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Pest Management

in the
Department of Biological Sciences
Faculty of Science

© Jaime M. Chalissery 2022
SIMON FRASER UNIVERSITY
Spring 2022

Copyright in this work rests with the author. Please ensure that any reproduction or re-use is done in accordance with the relevant national copyright legislation.

Declaration of Committee

Name:	**Jaime M. Chalissery**
Degree:	**Master of Pest Management**
Thesis title:	**Trail pheromone ecology of the pavement ant, *Tetramorium immigrans*, and the European fire ant, *Myrmica rubra***
Committee:	**Chair:** Chris Kennedy Professor, Biological Sciences
	Gerhard Gries Supervisor Professor, Biological Sciences
	Jenny Cory Committee Member Professor, Biological Sciences
	Robert J. Higgins Committee Member Associate Professor, Biological Sciences Thompson Rivers University
	Dong-Hwan Choe Examiner Associate Professor, Entomology University of California

Abstract

Foragers of many ant species deposit trail pheromones that guide nestmates to food resources. We identified 2-Methoxy-6-methylbenzoate ('MMMB') as the single-component trail pheromone of the pavement ant, *Tetramorium immigrans* (Hymenoptera: Formicidae). MMMB was sensed by worker antennae, induced trail following in laboratory bioassays, and effectively recruited nestmates to food baits in field settings. We deduced that only groups, and not individuals, of the European fire ant, *Myrmica rubra* (Hymenoptera: Formicidae) respond to pheromone trails. Furthermore, we determined that a 3-component pheromone blend comprising 3-ethyl-2,5-dimethylpyrazine ('EDP', the previously known trail pheromone component), (*Z*,*E*)-α-farnesene, and (*Z*,*E*)-α-homofarnesene, was superior to EDP in prompting (*i*) sustained trail-following behavior in laboratory bioassays and (*ii*) relatively faster recruitments of foraging ants to apple baits in a field experiment. All data combined provide impetus to develop synthetic trail pheromones coupled with lethal food baits as a tactic for integrated control of pest ants.

Keywords: Myrmicinae; *Tetramorium immigrans*; *Myrmica rubra*; communication; trail pheromone; field testing

Acknowledgements

Thank you to Dr. Chris Kennedy for agreeing to chair my defense; to Dr. Dong-Hwan Choe who agreed to attend as my external examiner providing his wealth of expertise and knowledge with ants, chemical ecology, and pest management; to Dr Jenny Cory and Dr. Rob J. Higgins for their continued expertise, guidance, and direction as members of my committee who have helped push my research into new areas, explore fascinating topics and questions, and thankfully telling me to stop doing things when we're in our committee meetings after seeing how excited about other research projects I would become.

Thank you to Dr. Gerhard Gries and Regine Gries for all their support, advice, and the opportunities they have given me starting in the lab after taking BISC 317 as an undergraduate research assistant. This has not only fully cemented my love of research and entomology, but the opportunities that came out of it inspired future goals and developed a plethora of skills. My time in the lab has helped me turn into the person I am today and help me realize what I want to do. Gerhard, thank you for your overwhelming optimism, it has been an amazing way to push me through difficult times in research when negative data strikes (though when you, Asim, and I get caught up in all the experiments its hard to stop making new projects) and always being available to stop by your office to chat about things has helped make my time doing research much easier. Regine, thank you for not only the amazing chemical wizardry you do, but also for being so jovial in lab and talking with everyone, it really makes working in there much easier always getting to chat with you or knowing how you always make time for people with your hectic schedule. You both have been irreplaceable for me and the research I have done and things that I have achieved, as well as being people I can talk to when things get rough in life or shit hits the fan with research. Thank you both very much, for helping me grow as a researcher and a person through my time in the lab.

There are many things during my undergraduate degree that pushed me towards grad school, but for sure wouldn't have started if not for me taking Insect Biology in Fall 2019 with Gerhard, Lydia Stepanovic, Tamara Trottier and Tiia Haapalainen. I will always remember that semester, classroom, fellow students, friends, and the teaching team – thank you Gerhard, Lydia, Tamara, and Tiia (& honestly all the students from BISC 317) for that amazing experience. Getting that push from Lydia to not only join the

lab as more than a volunteer and introducing me to other researchers (through puppysitting) really skyrocketed my confidence and was an amazing opportunity to continue down that new path.

This led to me doing multiple research courses with Danielle Hoefele, who introduced me to the devious *Myrmica rubra* and taught me so much about ants, pheromone communication and foraging strategies, as well as how to test all those things and make pretty presentations. Concurrently I did a research course with Heather Coatsworth in the Lowenberger lab, who not only let me puppysit Acorn, but also showed me how working with *Aedes aegyptii* gene knockdowns was extremely fascinating, developed new skills in wet lab work, and getting to enjoy being part of the Lowenberger lab. Thank you, Dan and Heats, for your amazing mentoring that has started my journey into research and eventually pursuing this Masters. You both are amazing researchers, mentors, people, and friends who taught me a lot and people I look up to.

The start of our friendship was hilarious with you asking me to try out your gym and the first session immediately starts with clinch work which resulted in our necks both being extremely sore the next day. Probably the most chaotic way to start being friends but lines up well how we interact now. I really appreciate all the time and effort you put into mentoring me when you didn't plan on having a student to babysit that term. From that point, all the collaborations and just generally becoming closer friends has made you one of the most impactful people on my time within the lab. The dynamic of you being my lab husband was a dumb start but really kept up since you helped me get through a lot, through advice, collaborations, and just being my biggest support within the lab and us starting up more of an "Ant Team" where we all (and our students) worked on things together and took over the Annex as our castle (I don't think we ever settled on a name though but all I think of right now is "Antnex" or "the Ant Hill" which is dumb). Thank you for always providing amazing criticism, breaking shit down, and for the talks we always have, though I miss the random brainstorming on transit after a Dageraad night – hurry up and come back from the Island. You are an amazing mentor and collaborator, and a top tier friend to both have smart and dumb moments which are always fun. Your weird brain capacity for ant facts, which displaces all other knowledge like where you leave all your things, and how you relate apples to turtles have always made doing research with you an amazing experience and something I will miss dearly,

though we're probably still going to bug the shit out of each other even once I am done. Final reminders, you still need to compete in the Dad-lympics and teach Clark how to drive, but thank you for everything bud.

Thank you to Dr. Howard Thistlewood, Dr. Carl Lowenberger, and Dr. Gerhard Gries for the opportunities to work in your labs, teaching me so much and guiding me through various research projects. Thank you Thistlewood Lab (Brigitte Rozema, Amanda Chaimberlain, Brian Muselle), Lowenberger Lab (Heather Coatsworth, Nicolas Salcedo Porras, Iman Baharmand, Soheil Saeidiborojeni, Lea Sanchez-Milde, William Shen, and Shir Yi Toh), Gries Lab (Regine Gries, Steve Takács, Santosh Kumar Alamsetti, Adam J. Blake, Asim Renyard, Elana Varner, Andi Fischer, Elton Ko, Sam Meraj, Emily Lemke, Kendal Singleton, Emmanuel Hung, Saif Nayani, Claire Gooding, Emma Kovacs, Warren Wong, Yonathan Uriel, Dan Hoefele, Dan Peach, Tamara Babcock, Josh Pol, Lydia Stepanovic, & all other students, USRAs, 49x's, volunteers) for the continual support throughout my time in the labs and beyond, always being amazing co-workers and friends that help make the work much more fun! Particular thanks to the chemistry wizards Santosh and Regine for their expertise that made this project and many of the other things I worked on succeed, and Adam and Asim for kindly bestowing me with but a smidge of their stats knowledge and being amazingly helpful with bouncing ideas around for figures, experiments, and stats.

A huge thank you to my (mostly fire) ant team: Asim Renyard, Kenza Zobadai, April Lin, Kennedy Hoven, Jenelle Breen, Jasper Li, Ranjot Bhandal, Claire Gooding, Jonathan Petrov, Madison J. Ardiel, Saif Nayani, Sebastian Damin, Jan Lee, Calla Pickett, Stephen Vaive, Julienne Coloma, Miele McGowan, Kelvin Lau, Kristy Lok, Kai Zhang, Srishti Kumar, Devon Rai, Jessica Chalissery, Rosemary Vayalikunnel, Kris Cu. I for sure wouldn't have gotten all this work and much more done without your help! All the time, effort, and ant stings if you got any were very much appreciated and mentoring or helping you have a good experience with your time in the lab was something I thoroughly enjoyed and look forward to see where you all go in the future – I know various have started or will be starting their own Master's degree, others working on various projects or continuing their undergraduate education, but to all of you, once again thank you, good luck in your futures, and always down for a chat! Also shout out to all the BISC 317 students I taught with Asim in 2019 Fall and with Kendal in 2021

Spring, was a pleasure to teach you all and some of the most fun times had even though they were super busy semesters!

I'd also like to thank Dr. Sean McCann for showing Asim and I all sorts of ants (as well as all the other insects, birds, and snakes we found), in particular the pavement ants, and giving guidance, resources, or just being available to chat about things. It has all been extremely helpful for getting certain parts of my research going and fully led to all the work with pavement ants. Thank you, Jason C. Roth for all the chats about ant keeping and for going scouting, was really fun to see the ants and get insights from everything you've learned so early on.

Thank you to my parents Vilson and Tessy Chalissery, and my brother Justin Chalissery, who have provided me with so much support and been met with my dumb frustration, keeping me going through things even when they don't understand what I'm doing or why I'm doing it. You all not only helped keep me going and kept me believing in myself when unsure about things. Your support and insight you provide from an outside perspective helped me get through this and are very appreciated and I hope you all understand a bit more about what I do from this presentation (since if the first time I've shown you one). You all do so much, and I forget to say it at times, but it means more than I can ever say. Also thank you to my extended family for all the support that you all have given me and the fun times of family gatherings, being there for each other, and just random check ins.

Thank you to my extremely dorky friends Max Almond, Asim Renyard, Adam Blake, Dan Peach, Josh Pol, Claire Gooding, Laurel Sleigh, and Gursimran Mahendru – who always want to get up to some glorious shenanigans that usually result in laughing at some shockingly spot on gif/photo edits that were made for some random thing we encountered. For sure the best way to be creative, improv with people, and try out random voices. Special thanks to Max for starting this whole descent into a hobby - getting me hooked on D&D, Critical Role, Not Another DnD Podcast, Dungeons and Daddies, and to those podcasts that got me through many long hours of bioassaying ants, analyzing videos, and being something to get lost in or cheer me up when it was needed. Thank you to James Huynh, Garrett Fournier, Alessandro Freeman, Julian Del Balso, Jonathan Rubianto, Sean Dance, Steve Sy, and Brandon Yau, who always are down for some gaming, hangouts, or random discord calls to watch shows. Thank you to

Don Wiggins, Pauline Deschodt, Elizabeth Steves, Heather Coatsworth, Kevin Colmenares-Di Maria, Ranah Chavoshi, Nicolas Salcedo Porras, Mikayla More O'Ferrall, Maddy Forrester, Devon Rai, and Kendal Singleton, for all the support, advice, hangouts, and always getting me outside to explore all sort of neature - always stopping to look at trees, insects, fungi, dogs, and anything else cause we're a bunch of nerdy biologists. Thank you to all the Dageraad faam that have made such an awesome place to go work in and hang out once we got tired of writing. For sure one of my favourite spots (through the introduction by Adam and Dan) to head to for working in a more fun environment and it has been great getting to know you all, especially Will and Michelle! Hopefully some more beers to be had there in the future! There are so many more people that have made my Masters a wonderful experience – thank you all! I opted to instead name a bunch of pets – you can't be mad at that: Thank you to all the animal owners/sitters who let me play with and/or watch your pets, they are amazing and thank you to all the animals too for being adorable and always making days better: Aches (Acorn), Ollie (Oliver), Luc (Lucy), Debs (Debu), Jack, Tills (Matilda/Tilly), Nelly, Perls (Perla), Bruno & Breeze, Giselle & Callie, Scotty, Toobs (Toby, & all other animal sent by Annie), Puck, Abbie, Bear, Quince & Roxy, Elliot, Zulu, Winston, Bacchus, Dante, Frankie & Elmer (& all the Eagle Acres kitties, dogs, goats, & cows), Caci, Ducky, Hank, Pico, Daisy, all the dogs at my field sites, the random physics dog, & all others dogs, insects, pets, etc. that I don't know the names or see in passing while doing work and in life.

To whoever is reading these long-winded acknowledgements, it has been cathartic to write it all out and wanted to thank all the people who have made this possible – so thank you for taking the time to read it. Finally, thank you to all the ants that I've had the pleasure (or pain – from stings or bites) to work with over all these years. You're way better than Diptera.

Table of Contents

List of Tables

List of Figures

Chapter 1.

The invasive pavement ant and European fire ant in British Columbia

Ants stand tall as one of the most fascinating taxonomic families of insects, originating at least 100 million years ago (115–185 mya; Brady et al., 2006; Crozier et al., 1997; Lach et al., 2010; Moreau et al., 2006; Perrichot et al., 2008). More than 12,500 species have been described and over 200 of those have established invasive populations (McGlynn, 1999; Hölldobler & Wilson, 1992; Holway et al., 2002). Advances in genetic analyses and taxonomy have facilitated species assignments and provided insights into population dynamics and invasion patterns of many species (Cordonnier et al., 2019; Wagner et al., 2017; Zhang et al., 2019). Many ant species are considered invasive but the main research focus is on major pest species such as the red imported fire ant, *Solenopsis invicta*, Argentine ant, *Linepithema humile*, tawny crazy ant, *Nylanderia fulva* (Lowe et al., 2000), and the European fire ant, *Myrmica rubra* (Robinson et al., 2013). In contrast, nuisance pest ants in urban settings, such as the pavement ant, *Tetramorium immigrans,* have received little research attention.

1.1. ***Tetramorium immigrans*** **(Santschi)**

Tetramorium immigrans is considered a minor pest both in their native and invaded ranges. Native in the Western Palaearctic, it was introduced to North America most likely in the 1800s (Brown, 1957; Flucher et al., 2021). In North America, pavement ants were referred to as *T. caespitum*, *T. caespitum var. immigrans*, *T. caespitum E*, and *T. species E*, exemplifying the difficulty in correct taxonomic assignment. Wagner et al. (2017) proposed a complete revision to the *Tetramorium caespitum* complex, ultimately showing that *T. immigrans* is the most common pavement ant in North America. It is present in British Columbia (BC; first specimen found in 2013 in Tsawwassen, with expansion to Vancouver Island and at least as far north as Kamloops; R.J. Higgins, pers. obs.), Ontario, and Quebec (Guénard, 2017), and in many areas of the USA (Zhang et al., 2019). The decryption of the *Tetramorium caespitum* complex has also provided

more information about the worldwide distribution of *T. immigrans* (Borowiec & Salata, 2021; Castracani et al., 2020; Salata et al., 2020; Samin et al., 2020; Zhang et al., 2019).

Tetramorium species thrive in urban environments and are aptly called pavement ants due to their habit of establishing colonies and tunnels under or between surfaces of stone and pavement. They also benefit from structural support and adequate shelter provided by plants (Cordonnier et al., 2019; Pećarević et al., 2010; Penick et al., 2015). *Tetramorium immigrans,* in particular, makes use of many types of habitat, such as rocky and sandy grasslands, rock walls, beaches (Wagner et al., 2017), urban pavement (Cordonnier et al., 2019), and inside homes (potentially nesting within wall cavities; R.J. Higgins, pers. obs.). When present, small debris mounds near nests make nest entrances clearly visible but they vary in size and location and may collapse after rain (Bruder & Gupta, 1972; Wagner et al., 2017). This variation and change in location between years make it difficult to determine whether multiple nest entrances in close proximity originate from a single colony.

Colonies are separated by territorial boundaries, as evident by massive battles that determine them (Cordonnier et al., 2021; Wheeler, 1927). Battles ensue from chance encounters of foragers from neighbouring colonies, with nestmates from both colonies being recruited until a swarm of ants blankets the boundary of a territory (Plowes, 2008). Nestmates are recruited through random walks of individuals, tandem running, or trail pheromones (Hoover et al., 2016; Planqué et al., 2010; Plowes, 2008). Initial costs associated with territorial battles could be recovered through sustained resources present in gained territory (Hoover et al., 2016; Plowes, 2008). Devoting efforts to ‘war’ may be optimal for established colonies with large numbers of workers but devoting efforts to increase colony size seems more appropriate for newly established colonies.

Start-up colonies grow slowly due to a single queen allocating resources and maintaining the first generation of brood, which is prone to cannibalism by both the queen and older larvae (Bruder & Gupta, 1972). By focusing on rapid production of relatively few and comparatively small workers (“nanitics”), the queen can quickly pass on the ‘chores’ of caring for the colony and foraging for resources. With increasingly more workers that provide external resources and care for the brood, colony growth booms with reduced cannibalism and longer development time for brood, leading to

larger individual brood and more brood overall (Bruder & Gupta, 1972). Alates occur from March to September, and form new colonies after their nuptial flights, but swarming of *T. immigrans* is not well studied (Cordonnier et al., 2021; Wagner et al., 2017).

As an urban nuisance pest, *T. immigrans* damages structural foundations and blemishes the aesthetics of pathways and driveways, the extent of which dependent upon ant colony size and the number of colonies in an area (Sano et al., 2018). When colonies establish near a house, foragers may enter the house in search of resources. Lacking a stinger that can pierce skin, worker ants do not pose a threat to humans. Tunneling is the most visible impact of *T. immigrans* but its myrmecochorous foraging also causes the spread of plant pests and can displace native ant species (Steiner et al., 2008; J.C. Roth, pers. obs.). By tending aphids for honeydew, *T. immigrans* hinders biocontrol tactics such as the release of predators or parasitoids (Bruder & Gupta, 1972; Stadler & Dixon, 2005; Völkl et al., 1999). Foragers of *T. immigrans* aggregate at plant lesions (JMC pers. obs.), possibly to obtain nutrients from the xylem or phloem. Studying their use of floral or extra-floral nectar, and other nutrient resources, may help us understand their nutritional requirements and thus their role in urban arthropod communities.

1.2. *Myrmica rubra* (Linnaeus)

Myrmica rubra is native to Europe and central Asia where it is a nuisance but not a major pest. Following its arrival in Massachusetts (USA) early in the 20th century (Groden et al., 2005), *M. rubra* has quickly spread, likely through plant material being shipped from Europe or from previously invaded regions in North America (Groden et al., 2005). *Myrmica rubra* has now been reported in Eastern and Western North America (USA: six northeastern states and Washington state; Canada: BC, Ontario and all provinces eastward) and is viewed as a major pest ant (Naumann & Higgins, 2015). The presence of *M. rubra* in Western North America is likely due to a separate introduction from Europe or Asia rather than the result of spreading from the East coast (Invasive Species Council of BC, 2013). In BC, *M. rubra* has likely been present since the late 20th century. Increased notice of *M. rubra* throughout North America may be due to increased public knowledge about this ant species or population growth supported by new adaptations or increasing global temperature (Groden et al., 2005; Invasive Species Council of BC, 2013; Naumann & Higgins, 2015).

With multiple reproductive queens and nests per colony, *M. rubra* is both polygynous and polydomous (Arevalo & Groden, 2007). On average, there are 15 queens in each colony, but as many as 670 queens have been observed in a single colony (Groden et al., 2005). Eradication of colonies is difficult, because they are subterranean with only small entrance holes that may hidden under the base of trees, fallen logs, leaf litter, moss, raised garden beds, or paving blocks or rocks (Higgins, 2015, Chalissery, pers. obvs.). Moreover, every single queen must be eliminated to eradicate a colony (Groden et al., 2005). As *M. rubra* prefers environments with high humidity, gardens are prime habitats but lawns, wetlands, deciduous forests, coniferous forests, golf courses, and other urban environments are also inhabited (Groden et al., 2005; Higgins, 2015; Robinson et al., 2013). In its native range in Europe and central Asia, *M. rubra* disperses through both nuptial flights and 'budding'. Nuptial flights usually take place in late summer, with alates (winged reproductive males and females) forming mating swarms. Mated queens then overwinter alone or in groups, whereas mated males die due to a lack of energy (Groden et al., 2005). Budding takes place throughout the foraging season, involving wingless queens (singly or in groups) leaving their 'parent' colony with a founding party (a group of workers and potentially some brood) and starting their own colony elsewhere or joining a pre-existing one. In its invaded North American range, *M. rubra* still produces winged queens and males (Groden et al., 2005) but only one nuptial flight has been recorded thus far, with no females present (Hicks, 2012). This lack of nuptial flights leads to extremely high nest densities (Groden et al., 2005; Robinson et al., 2013).

In Europe (and possibly also in North America), both queens and workers of *M. rubra* produce different types of brood (eggs and subsequent larvae). The brood produced by queens in the summer becomes worker ants within the same season, whereas the winter brood overwinters and becomes adult ants the following season due to larval and pupal development rates being slower and less dependent on temperature (Kipyatkov et al., 2005; Kipyatkov & Lopatina, 1997). Worker ants lay trophic eggs that feed larvae and queens, and they produce reproductive brood that yields male ants (Brian & Rigby, 1978).

Worker ants of *M. rubra* seek a wide variety of food sources that provide carbohydrates, proteins, and lipids to the colony (Bologna & Detrain, 2019; Brian & Abbott, 1977), with foraging efforts and food preferences changing in accordance with

the presence of brood. As omnivores, these ants meet their dietary requirements by preying on invertebrates, exploiting (extra)floral nectar of plants, and scavenging on food discarded by humans or animals. Workers of *M. rubra* also tend hemipterans, like aphids, to obtain carbohydrate-rich honeydew, and they collect seed elaiosomes which are rich in proteins and lipids (Fischer et al., 2005; Stadler & Dixon, 2005; Völkl et al., 1999).

Due to its aggressive behaviour and extremely large populations, *M. rubra* has become the predominant ant species in infested areas, strongly affecting native arthropods. Across invaded habitats, *M. rubra* made up 99.99% of the ant fauna captured in pitfall traps, accounted for lower arthropod biodiversity, was 10- to 1300-fold more abundant than all other ants combined in non-infested sites (British Columbia; Naumann & Higgins, 2015), and colonized most food baits more quickly than native ant species (Maine; Garnas et al., 2014). The presence of *M. rubra* also impacted the reproductive success of the European herring gull, *Larus argentatus* (DeFisher & Bonter, 2013). The presence of *M. rubra* disrupted nest site selection by these birds, caused erratic incubation behaviour, slowed chick growth rates, and increased predation on nestlings (DeFisher & Bonter, 2013). There are various other examples of ants, particularly fire ants, affecting bird species (Allen et al., 1995; Ridlehuber, 1982). As *myrmecochorous* foragers, *M. rubra* workers greatly contribute to plant seed dispersal (Detrain & Bologna, 2019) but their close association with invasive plants, such as Himalayan blackberry, *Rubus armeniacus,* promotes the spread of unwanted flora (Meadley-Dunphy et al., 2020, JMC, pers. obvs.). Moreover, aphid farming by *M. rubra* causes problems in botanical gardens and greenhouses as worker ants protect aphids from biocontrol agents (Stadler & Dixon, 2005; Völkl et al., 1999).

The presence and aggressive behaviour of *M. rubra* are problematic in gardens and recreational areas. Workers of *M. rubra* sting anything that disrupts their foraging activity or their colony. Hundreds of nestmates that are recruited by alarm pheromone may swarm and sting the ‘perpetrator’. The pain inflicted by stings resembles that caused by mild wasp stings or stinging nettle (Higgins, pers. comm.), and the resulting itchiness persists for an hour up to several days (JMC, pers. obvs.). There is a risk of anaphylactic shock and potential hospitalization depending on a person’s sensitivity to the ant venom and the number of stings they received. Saltman (2016) reported BC’s first case of anaphylaxis from *M. rubra* stings. With human activities and trade being the

main modes of *M. rubra* dispersal, infestations are likely to occur in gardens and recreational areas due to deliveries of soil infested with *M. rubra*. Once established in a habitat, *M. rubra* colonies are exceedingly difficult to eradicate. New infestations of *M. rubra* are not candidly reported because of concerns about declining property values and fewer visitors to recreational parks (Higgins, pers. comm.). A 2012-assessment projected the annual economic loss caused by *M. rubra* in BC (if it were to spread across all inhabitable areas) to range between $100 – 160 million CAD (Robinson et al., 2013).

1.3. **Trail Pheromones**

Chemical signals (pheromones) are essential in ant communication (Morgan, 2009). Ant colonies use pheromonal communication to deploy their workforce effectively for specific tasks such as cooperative brood care, nest defense and foraging (Hölldobler & Wilson, 1990). Pheromones broadcast information context-specifically. For example, when colonies are under attack, nestmates emit an alarm pheromone that mediates concerted colony defense (Blum, 1969). Trail pheromones, in contrast, serve in the context of foraging (Morgan, 2009).

Like many other ants, *Tetramorium* and *Myrmica* colonies recruit nestmates to food sources via trail pheromones that foraging ants deposit as chemical orientation guidelines for nestmates (Beckers et al., 1990; Czaczkes et al., 2015; Morgan, 2009). To date, trail pheromones are known for four *Tetramorium* species which differ in the number of trail pheromone components and their molecular structure (Table 1.1). In all four *Tetramorium* species studied to date, the poison gland produces and secretes the trail pheromone (Attygalle & Morgan, 1983; Jackson et al., 1990; Morgan & Ollett, 1987; Nakamura et al., 2019), as has been shown for more than 30 myrmicine ant species (Cerdá et al., 2014; Morgan, 2009). *Tetramorium immigrans* has only recently been delineated from *T. caespitum*, and thus its trail pheromone has not yet been studied. My objectives for Chapter 2 were to identify the glandular source and chemical component of the trail pheromone of *T. immigrans*, and test trail following activity of a synthetic version for individual *T. immigrans* workers.

At least eight *Myrmica* species share 3-ethyl-2,5-dimeythylpyrazine as a trail pheromone component (Table 1.1) and some of these species respond to poison gland extracts (containing trail pheromone components) from heterospecific ants (Cammaerts

et al., 1981; Evershed et al., 1982). In laboratory bioassays, groups of *M. rubra* worker ants, but not single ants, readily followed trails of 3-ethyl-2,5-dimethylpyrazine (Evershed et al., 1982; Chalissery et al., 2019, Hoefele et al., 2020, 2021), supporting the concept (Cammaerts-Tricot, 1978; Vander Meer et al., 1990) that physical activation or motivation by nestmates is required to induce trail following behavior in *M. rubra.* This concept, while applicable to *M. rubra,* contrasts reports of single-ant trail-following behavior observed in other species of ants (e.g., Chalissery et al., 2019, 2021; Renyard et al., 2019). Evidence that groups of *M. rubra* worker ants in field trials did not readily follow experimental trails of 3-ethyl-2,5-dimeythylpyrazine (Hoefele et al., pers. comm.) further implies that the *M. rubra* trail pheromone is complex, containing components other than just 3-ethyl-2,5-dimeythylpyrazine. These components may be produced in, and released from, the poison gland, the Dufour's gland, or both (Cerdá et al., 2014). Poison or Dufour's gland extracts of *M. rubra* indeed contain additional compounds (Attygalle, et al., 1983; Attygalle, et al., 1983; Morgan & Wadhams, 1972) but their potential functional role as trail pheromone components has not yet been tested. My objectives in Chapter 3 were to reanalyze poison and Dufour's glands of *M. rubra* for the presence of additional candidate trail pheromone components, and to test these components for their ability to enhance the pheromonal activity of 3-ethyl-2,5-dimeythylpyrazine in trail following bioassays with *M. rubra* workers tested singly or in groups.

1.4. **References**

Adams, R. M. M., Wells, R. L., Yanoviak, S. P., Frost, C. J., & Fox, E. G. P. (2020). Interspecific eavesdropping on ant chemical communication. *Frontiers in Ecology and Evolution*, *8*, 1–22. https://doi.org/10.3389/fevo.2020.00024

Allen, C. R., Lutz, R. S., & Demarais, S. (1995). Red Imported Fire Ant Impacts on Northern Bobwhite Populations. *Ecological Applications*, *5*(3), 632–638. https://doi.org/10.2307/1941972

Arevalo, H. A., & Groden, E. (2007). European fire ant, red ant (suggested common names), *Myrmica rubra* Linnaeus (Insecta: Hymenoptera: Formicidae: Myrmicinae). *University of Florida, Institute of Food and Agricultural Sciences, Florida Cooperative Extension Publication EENY-410*, 1–5.

Attygalle, A. B., Cammaerts, M. C., & Morgan, E. D. (1983). Dufour gland secretions of *Myrmica rugulosa* and *Myrmica schencki* workers. *Journal of Insect Physiology*, *29*(1), 27–32. https://doi.org/10.1016/0022-1910(83)90102-6

Attygalle, A. B., Evershed, R. P., Morgan, E. D., & Cammaerts, M. C. (1983). Dufour gland secretions of workers of the ants *Myrmica sulcinodis* and *Myrmica lobicornis*, and comparison with six other species of *Myrmica*. *Insect Biochemistry*, *13*(5), 507–512. https://doi.org/10.1016/0020-1790(83)90009-4

Attygalle, A. B., & Morgan, E. D. (1983). Identification of trail pheromone of the ant *Tetramorium caespitum* L. (Hymenoptera: Myrmicinae). *Naturwissenschaften*, *70*, 364–365. https://doi.org/10.1007/BF00990315

Baur, B., Storch, K., Martz, K. E., Goettert, M. I., Richters, A., Rauh, D., & Laufer, S. A. (2013). Metabolically Stable Dibenzo[*b*,*e*]oxepin-11(6 *H*)-ones as Highly Selective p38 MAP Kinase Inhibitors: Optimizing Anti-Cytokine Activity in Human Whole Blood. *Journal of Medicinal Chemistry*, *56*(21), 8561–8578. https://doi.org/10.1021/jm401276h

Beckers, R., Deneubourg, J. L., Goss, S., & Pasteels, J. M. (1990). Collective decision making through food recruitment. *Insectes Sociaux*, *37*(3), 258–267. https://doi.org/10.1007/BF02224053

Bestmann, H. J., Kern, F., Schäfer, D., & Witschel, M. C. (1992). 3,4-Dihydroisocoumarins, a new class of ant trail pheromones. *Angewandte Chemie International Edition in English*, *31*(6), 795–796. https://doi.org/10.1002/anie.199207951

Billen, J., & Morgan, E. D. (1998). Pheromone communication in social insects: Sources and secretions. In *Pheromone Communication in Social Insects* (pp. 3–33). Westview Press.

Blum, M. S. (1969). Alarm pheromones. *Annual Review of Entomology*, *14*(1), 57–80. https://doi.org/10.1146/annurev.en.14.010169.000421

Bologna, A., & Detrain, C. (2019). Larvae promote a decline in the harvesting of myrmecochorous seeds by *Myrmica rubra* ants. *Insectes Sociaux*, *66*(3), 453–461. https://doi.org/10.1007/s00040-019-00706-y

Borowiec, L., & Salata, S. (2021). Notes on ants (Hymenoptera: Formicidae) of Corfu Island, Greece. *Annals of the Upper Silesian Museum in Bytom, Entomology*, *30*, 1–19. https://doi.org/10.5281/ZENODO.4550403

Brady, S. G., Schultz, T. R., Fisher, B. L., & Ward, P. S. (2006). Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proceedings of the National Academy of Sciences*, *103*(48), 18172–18177. https://doi.org/10.1073/pnas.0605858103

Brian, M. V., & Abbott, A. (1977). The control of food flow in a society of the ant *Myrmica rubra* L. *Animal Behaviour*, *25*, 1047–1055. https://doi.org/10.1016/0003-3472(77)90055-0

Brian, M. V., & Rigby, C. (1978). The trophic eggs of *Myrmica rubra* L. *Insectes Sociaux*, *25*(1), 89–110. https://doi.org/10.1007/BF02224488

Brown, W. L. J. (1957). Is the ant genus *Tetramorium* native in North America? *Breviora*, *72*, 1–8.

Bruder, K. W., & Gupta, A. P. (1972). Biology of the Pavement Ant, Tetramorium caespitum (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, *65*(2), 358–367. https://doi.org/10.1093/aesa/65.2.358

Buczkowski, G., & Richmond, D. S. (2012). The effect of urbanization on ant abundance and diversity: A temporal examination of factors affecting biodiversity. *PLoS ONE*, *7*(8), e41729. https://doi.org/10.1371/journal.pone.0041729

Cammaerts, M. C., Evershed, R. P., & Morgan, E. D. (1981). Comparative study of the mandibular gland secretion of four species of Myrmica ants. *Journal of Insect Physiology*, *27*(4), 225–231. https://doi.org/10.1016/0022-1910(81)90055-X

Cammaerts, M. C., Inwood, M. R., Morgan, E. D., Parry, K., & Tyler, R. C. (1978). Comparative study of the pheromones emitted by workers of the ants *Myrmica rubra* and *Myrmica scabrinodis*. *Journal of Insect Physiology*, *24*(3), 207–214. https://doi.org/10.1016/0022-1910(78)90036-7

Cammaerts, M., Evershed, R., & Morgan, E. (1981). Comparative study of the Dufour gland secretions of workers of four species of *Myrmica* ants. *Journal of Insect Physiology*, *27*(1), 59–65.

Cammaerts-Tricot, M. C. (1978). Recruitment to food in *Myrmica rubra* L. *Biology of Behaviour*, *4*, 159–172.

Castracani, C., Spotti, F. A., Schifani, E., Giannetti, D., Ghizzoni, M., Grasso, D. A., & Mori, A. (2020). Public Engagement Provides First Insights on Po Plain Ant Communities and Reveals the Ubiquity of the Cryptic Species Tetramorium immigrans (Hymenoptera, Formicidae). *Insects*, *11*(10), 678. https://doi.org/10.3390/insects11100678

Cerdá, X., van Oudenhove, L., Bernstein, C., & Boulay, R. R. (2014). A list of and some comments about the trail pheromones of ants. *Natural Product Communications*, *9*(8), 1934578X1400900. https://doi.org/10.1177/1934578X1400900813

Chalissery, J. M., Gries, R., Alamsetti, S. K., Ardiel, M. J., & Gries, G. (2022). Identification of the trail pheromone of the pavement ant *Tetramorium immigrans* (Hymenoptera: Formicidae). *Journal of Chemical Ecology*, *48*(3), 302–311. https://doi.org/10.1007/s10886-021-01317-3

Chalissery, J. M., Renyard, A., Gries, R., Hoefele, D., Alamsetti, S. K., & Gries, G. (2019). Ants sense, and follow, trail pheromones of ant community members. *Insects*, *10*(11), 383. https://doi.org/10.3390/insects10110383

Choe, D.-H., Villafuerte, D. B., & Tsutsui, N. D. (2012). Trail Pheromone of the Argentine Ant, Linepithema humile (Mayr) (Hymenoptera: Formicidae). *PLoS ONE*, *7*(9), e45016. https://doi.org/10.1371/journal.pone.0045016

Cordonnier, M., Gibert, C., Bellec, A., Kaufmann, B., & Escarguel, G. (2019). Multi-scale impacts of urbanization on species distribution within the genus *Tetramorium*. *Landscape Ecology*, *34*(8), 1937–1948. https://doi.org/10.1007/s10980-019-00842-7

Cordonnier, M., Kaufmann, B., Simon, L., Escarguel, G., & Mondy, N. (2021). Discrimination of conspecifics from heterospecifics in a hybrid zone: Behavioral and chemical cues in ants. *Insect Science*, 1744-7917.12915. https://doi.org/10.1111/1744-7917.12915

Crozier, R. H., Jermiin, L. S., & Chiotis, M. (1997). Molecular Evidence for a Jurassic Origin of Ants. *Naturwissenschaften*, *84*(1), 22–23. https://doi.org/10.1007/s001140050341

Czaczkes, T. J., Grüter, C., & Ratnieks, F. L. W. (2015). Trail pheromones: An integrative view of their role in social insect colony organization. *Annual Review of Entomology*, *60*(1), 581–599. https://doi.org/10.1146/annurev-ento-010814-020627

DeFisher, L. E., & Bonter, D. N. (2013). Effects of invasive European fire ants (*Myrmica rubra*) on herring gull (*Larus argentatus*) reproduction. *PLoS ONE*, *8*(5), e64185. https://doi.org/10.1371/journal.pone.0064185

Detrain, C., & Bologna, A. (2019). Impact of seed abundance on seed processing and dispersal by the red ant *Myrmica rubra*: Seed dispersal by ants. *Ecological Entomology*, *44*(3), 380–388. https://doi.org/10.1111/een.12713

Detrain, C., & Deneubourg, J.-L. (2008). Collective decision-making and foraging patterns in ants and honeybees. In *Advances in insect physiology* (Vol. 35, pp. 123–173). Elsevier. https://doi.org/10.1016/S0065-2806(08)00002-7

Evershed, R. P., Morgan, E. D., & Cammaerts, M. C. (1982). 3-Ethyl-2,5-dimethylpyrazine, the trail pheromone from the venom gland of eight species of *Myrmica* ants. *Insect Biochemistry*, *12*(4), 383–391. https://doi.org/10.1016/0020-1790(82)90035-X

Fedoseeva, E. B. (2015). A technological approach to the description of group foraging in the ant *Myrmica rubra*. *Entomological Review*, *95*(8), 984–999. https://doi.org/10.1134/S0013873815080060

Fischer, R. C., Ölzant, S. M., Wanek, W., & Mayer, V. (2005). The fate of *Corydalis cava* elaiosomes within an ant colony of *Myrmica rubra*: Elaiosomes are preferentially fed to larvae. *Insectes Sociaux*, *52*(1), 55–62. https://doi.org/10.1007/s00040-004-0773-x

Flucher, S. M., Krapf, P., Arthofer, W., Suarez, A. V., Crozier, R. H., Steiner, F. M., & Schlick-Steiner, B. C. (2021). Effect of social structure and introduction history on genetic diversity and differentiation. *Molecular Ecology*, mec.15911. https://doi.org/10.1111/mec.15911

Garnas, J., Groden, E., & Drummond, F. A. (2014). Mechanisms of competitive displacement of native ant fauna by invading *Myrmica rubra* (Hymenoptera: Formicidae) populations. *Environmental Entomology*, *43*(6), 1496–1506. https://doi.org/10.1603/EN14079

Greenberg, L., & Klotz, J. H. (2000). Argentine ant (Hymenoptera: Formicidae) trail pheromone enhances consumption of liquid sucrose solution. *Journal of Economic Entomology*, *93*(1), 119–122. https://doi.org/10.1603/0022-0493-93.1.119

Gries, R., Herk, W., Alamsetti, S. K., Catton, H., Meers, S., Otani, J., & Gries, G. (2022). (*Z* ,*E*)-α-Farnesene—Sex pheromone component of female click beetle *Selatosomus aeripennis destructor* with intra- and inter-sexual communication function. *Entomologia Experimentalis et Applicata*, eea.13142. https://doi.org/10.1111/eea.13142

Gries, R., Khaskin, G., Gries, G., Bennett, R. G., King, G. G. S., Morewood, P., Slessor, K. N., & Morewood, W. D. (2002). (Z,Z)-4,7-Tridecadien-(*S*)-2-yl acetate: Sex pheromone of Douglas-fir cone gall midge, *Contarinia oregonensis*. *Journal of Chemical Ecology*, *28*, 2283–2297. https://doi.org/10.1023/A:1021005517389

Groden, E., Drummond, F. A., Garnas, J., & Franceour, A. (2005). Distribution of an Invasive Ant, *Myrmica rubra* (Hymenoptera: Formicidae), in Maine. *JOURNAL OF ECONOMIC ENTOMOLOGY*, *98*(6), 1774–1784.

Guénard, B. (2017). The global ant biodiversity informatics (GABI) database: Synthesizing data on the geographic distribution of ant species (Hymenoptera: Formicidae). *Myrmecological News*, *24*, 83–89. https://doi.org/10.25849/myrmecol.news_024:083

Helms, J. A., Roeder, K. A., Ijelu, S. E., Ratcliff, I., & Haddad, N. M. (2021). Bioenergy landscapes drive trophic shifts in generalist ants. *Journal of Animal Ecology*, *90*(3), 738–750. https://doi.org/10.1111/1365-2656.13407

Hicks, B. J. (2012). How does *Myrmica rubra* (Hymenoptera: Formicidae) disperse in its invasive range? Record of male-only swarming flights from Newfoundland. *Myrmecological News*, *16*, 31–34.

Hicks, B. J., & Marshall, H. D. (2018). Population structure of *Myrmica rubra* (Hymeno-ptera: Formicidae) in part of its invasive range revealed by nuclear DNA markers and aggression analysis. *Myrmecological News*, *28*, 35–43. https://doi.org/10.25849/myrmecol.news_028:035

Higgins, R. (2015, April 22). *The European Fire Ant in British Columbia: Identification and Approaches to Control*.

Hoefele, D., Chalissery, J. M., Gries, R., & Gries, G. (2020). Effects of trail pheromone purity, dose, and type of placement on recruiting European fire ants, *Myrmica rubra*, to food baits. *Journal of the Entomological Society of British Columbia*, *117*, 31–41.

Hoefele, D., Chalissery, J. M., Renyard, A., & Gries, G. (2021). Experimentally guided development of a food bait for European fire ants. *Entomologia Experimentalis et Applicata*, *169*, 780–791. https://doi.org/10.1111/eea.13053

Hölldobler, B., Oldham, N. J., Morgan, E. D., & König, W. A. (1995). Recruitment pheromones in the ants *Aphaenogaster albisetosus* and *A. cockerelli* (Hymenoptera: Formicidae). *Journal of Insect Physiology*, *41*(9), 739–744. https://doi.org/10.1016/0022-1910(95)00041-R

Hölldobler, B., & Wilson, E. O. (1990). *The ants*. Belknap Press of Harvard University Press.

Holway, D. A., Lach, L., Suarez, A. V., Tsutsui, N. D., & Case, T. J. (2002). The Causes and Consequences of Ant Invasions. *Annual Review of Ecology and Systematics*, *33*(1), 181–233. https://doi.org/10.1146/annurev.ecolsys.33.010802.150444

Hoover, K. M., Bubak, A. N., Law, I. J., Yaeger, J. D. W., Renner, K. J., Swallow, J. G., & Greene, M. J. (2016). The organization of societal conflicts by pavement ants *Tetramorium caespitum*: An agent-based model of amine-mediated decision making. *Current Zoology*, *62*(3), 277–284. https://doi.org/10.1093/cz/zow041

Invasive Species Council of BC. (2013). *European fire ant in BC: 2012 field results and planning forward workshop* (pp. 1–19) [Report based on the workshop for European fire ant]. BC Inter-Ministry Invasive Species Working Group.

Jackson, B. D., Keegans, S. J., Morgan, E. D., Cammaerts, M.-C., & Cammaerts, R. (1990). Trail pheromone of the ant *Tetramorium meridionale*. *Naturwissenschaften*, *77*, 294–296.

Keeling, C. I., Plettner, E., & Slessor, K. N. (2004). Hymenopteran semiochemicals. In S. Schulz (Ed.), *The chemistry of pheromones and other semiochemicals I* (Vol. 239, pp. 133–177). Springer Berlin Heidelberg. https://doi.org/10.1007/b95452

Kipyatkov, V. E., & Lopatina, E. B. (1997). Experimental study of seasonal cycle of rapid brood production in the ants *Myrmica rubra* L. and *M. ruginodis* Nyl. From two different latitudes. *Proceedings of the International Colloquia on Social Insects*, *3–4*, 195–206.

Kipyatkov, V. E., Lopatina, E., & Imamgaliev, A. (2005). Duration and thermal reaction norms of development are significantly different in winter and summer brood pupae of the ants *Myrmica rubra* LINNAEUS, 1758 and *M. ruginodis* NYLANDER, 1846 (Hymenoptera: Formicidae). *Myrmecological News*, *7*, 69–76.

Kolay, S., Boulay, R., & d'Ettorre, P. (2020). Regulation of ant foraging: A review of the role of information use and personality. *Frontiers in Psychology*, *11*, 734. https://doi.org/10.3389/fpsyg.2020.00734

Lach, L., Parr, C. L., & Abbott, K. L. (Eds.). (2010). *Ant ecology*. Oxford University Press.

Lehue, M., & Detrain, C. (2020). Foraging through multiple nest holes: An impediment to collective decision-making in ants. *PLOS ONE*, *15*(7), e0234526. https://doi.org/10.1371/journal.pone.0234526

Lehue, M., Detrain, C., & Collignon, B. (2020). Nest entrances, spatial fidelity, and foraging patterns in the red ant *Myrmica rubra*: A field and theoretical study. *Insects*, *11*(5), 317. https://doi.org/10.3390/insects11050317

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). *100 of the world's worst invasive alien species: A selection from the global invasive species database.* (Vol. 12). Invasive Species Specialist Group.

McGlynn, T. P. (1999). The worldwide transfer of ants: Geographical distribution and ecological invasions. *Journal of Biogeography*, *26*(3), 535–548. https://doi.org/10.1046/j.1365-2699.1999.00310.x

Meadley-Dunphy, S. A., Prior, K. M., & Frederickson, M. E. (2020). Invasive ants disperse seeds farther than native ants, affecting the spatial pattern of seedling recruitment and survival. *Oecologia*, *192*(1), 119–132. https://doi.org/10.1007/s00442-019-04553-3

Moreau, C. S., Bell, C. D., Vila, R., Archibald, S. B., & Pierce, N. E. (2006). Phylogeny of the Ants: Diversification in the Age of Angiosperms. *Science*, *312*(5770), 101–104. https://doi.org/10.1126/science.1124891

Morgan, E. D. (2009). Trail pheromones of ants. *Physiological Entomology*, *34*(1), 1–17. https://doi.org/10.1111/j.1365-3032.2008.00658.x

Morgan, E. D., Jackson, B. D., Ollett, D. G., & Sales, G. W. (1990). Trail pheromone of the ant *Tetramorium impurum* and model compounds: Structure-activity comparisons. *Journal of Chemical Ecology*, *16*(12), 3493–3510. https://doi.org/10.1007/BF00982113

Morgan, E. D., & Ollett, D. G. (1987). Methyl 6-methylsalicylate, trail pheromone of the ant *Tetramorium impurum*. *Naturwissenschaften*, *74*(12), 596–597. https://doi.org/10.1007/BF00368520

Morgan, E. D., & Wadhams, L. J. (1972). Chemical constituents of Dufour's gland in the ant, *Myrmica rubra*. *Journal of Insect Physiology*, 1125–1135.

Muscedere, M. L., Johnson, N., Gillis, B. C., Kamhi, J. F., & Traniello, J. F. A. (2012). Serotonin modulates worker responsiveness to trail pheromone in the ant *Pheidole dentata*. *Journal of Comparative Physiology A*, *198*(3), 219–227. https://doi.org/10.1007/s00359-011-0701-2

Nakamura, T., Harada, K., & Akino, T. (2019). Identification of methyl 6-methylsalicylate as the trail pheromone of the Japanese pavement ant *Tetramorium tsushimae* (Hymenoptera: Formicidae). *Applied Entomology and Zoology*, *54*(3), 297–305. https://doi.org/10.1007/s13355-019-00626-0

Naumann, K., & Higgins, R. J. (2015). The European fire ant (Hymenoptera: Formicidae) as an invasive species: impact on local ant species and other epigaeic arthropods. *The Canadian Entomologist*, *147*(5), 592–601. https://doi.org/10.4039/tce.2014.69

Naumann, K., Sá, M. M. D., Lewis, E., & Noronha, R. (2017). Supercolonies of the invasive ant, *Myrmica rubra* (Hymenoptera: Formicidae) in British Columbia, Canada. *Journal of the Entomological Society of British Columbia*, *114*, 56–64.

Nishisue, K., Koyama, S., & Satoh, T. (2020). Identification of the Argentine ant *Linepithema humile* (Hymenoptera: Formicidae) using an artificially synthesized trail pheromone and its effects on native Japanese ants. *Applied Entomology and Zoology*, *55*(1), 141–147. https://doi.org/10.1007/s13355-019-00663-9

Nishisue, K., Sunamura, E., Tanaka, Y., Sakamoto, H., Suzuki, S., Fukumoto, T., Terayama, M., & Tatsuki, S. (2010). Long-term field trial to control the invasive Argentine ant (Hymenoptera: Formicidae) with synthetic trail pheromone. *JOURNAL OF ECONOMIC ENTOMOLOGY*, *103*(5), 1784–1789. https://doi.org/10.1603/EC10008

Ondet, P., Lempenauer, L., Duñach, E., & Lemière, G. (2016). Bismuth(III) triflate-catalysed tandem cyclisations towards complex polycyclic ethers. *Organic Chemistry Frontiers*, *3*(8), 999–1003. https://doi.org/10.1039/C6QO00212A

Orlova, M., & Amsalem, E. (2019). Context matters: Plasticity in response to pheromones regulating reproduction and collective behavior in social Hymenoptera. *Current Opinion in Insect Science*, *35*, 69–76. https://doi.org/10.1016/j.cois.2019.07.004

Pasteels, J. M., Verhaeghe, J. C., Braekman, J. C., Daloze, D., & Tursch, B. (1980). Caste-dependent pheromones in the head of the ant *Tetramorium caespitum*. *Journal of Chemical Ecology*, *6*, 467–472.

Pećarević, M., Danoff-Burg, J., & Dunn, R. R. (2010). Biodiversity on Broadway—Enigmatic Diversity of the Societies of Ants (Formicidae) on the Streets of New York City. *PLoS ONE*, *5*(10), e13222. https://doi.org/10.1371/journal.pone.0013222

Penick, C. A., Savage, A. M., & Dunn, R. R. (2015). Stable isotopes reveal links between human food inputs and urban ant diets. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1806), 20142608. https://doi.org/10.1098/rspb.2014.2608

Perrichot, V., Lacau, S., Néraudeau, D., & Nel, A. (2008). Fossil evidence for the early ant evolution. *Naturwissenschaften*, *95*(2), 85–90. https://doi.org/10.1007/s00114-007-0301-8

Planqué, R., van den Berg, J. B., & Franks, N. R. (2010). Recruitment strategies and colony size in ants. *PLoS ONE*, *5*(8), e11664. https://doi.org/10.1371/journal.pone.0011664

Plowes, N. J. R. (2008). *Self organized conflicts in territorial ants*. University of Connecticut.

Ramaiah, P., Pegram, J. J., & Millar, J. G. (1995). A short synthesis of (3*Z*,6*E*)-α-farnesene. *The Journal of Organic Chemistry*, *60*(19), 6211–6213. https://doi.org/10.1021/jo00124a046

Rawat, D. S., & Gibbs, R. A. (2002). Synthesis of 7-Substituted Farnesyl Diphosphate Analogues. *Organic Letters*, *4*(18), 3027–3030. https://doi.org/10.1021/ol026176i

Reim, S., Lau, M., Adeel, M., Hussain, I., Yawer, M., Riahi, A., Ahmed, Z., Fischer, C., Reinke, H., & Langer, P. (2009). Synthesis of biaryls, fluorenones,cyclopenta[*def*]phenanthren-4-ones, and benzophenones based on formal [3+3] cyclocondensationsof 1,3-bis(silyloxy)buta-1,3-dienes with 3-(silyloxy)-2-en-1-ones. *Synthesis*, *2009*(03), 445–463. https://doi.org/10.1055/s-0028-1083309

Renyard, A., Alamsetti, S. K., Gries, R., Munoz, A., & Gries, G. (2019). Identification of the trail pheromone of the carpenter ant *Camponotus modoc*. *Journal of Chemical Ecology*, *45*(11–12), 901–913. https://doi.org/10.1007/s10886-019-01114-z

Ridlehuber, K. T. (1982). Fire Ant Predation on Wood Duck Ducklings and Pipped Eggs. *The Southwestern Naturalist*, *27*(2), 222. https://doi.org/10.2307/3671153

Robinson, D. C. E., Knowler, D., Kyobe, D., & de la Cueva Bueno, P. (2013). Preliminary damage estimates for selected invasive fauna in B.C. In *Ecosystems Branch B.C. Ministry of Environment* (p. 62). Ecosystems Branch B.C. Ministry of Environment. http://preventinganinvasion.psmfc.org/wp-content/uploads/2013/05/InvasiveFauna_EconomicImpacts_Prelim-BC_2013.pdf

Salata, S., Borowiec, L., & Trichas, A. (2020). *Review of ants (Hymenoptera: Formicidae) of Crete, with keys to species determination and zoogeographical remarks*. https://doi.org/10.5281/ZENODO.3738001

Saltman, J. (2016). B.C.'s First Case of Anaphylaxis from European Fire Ant Stings Confirmed. *Vancouver Sun*. http://vancouversun.com/news/local-news/b-c-s-first-case-of-anaphylaxis-fromeuropean- fire-ant-stings-confirmed

Samin, N., Yusupov, Z., Navaeian, M., & Sakenin, H. (2020). A contribution to ants (Hymenoptera: Formicidae) from North and Northwestern regions of Iran. *Natura Somogyiensis*, *35*, 29–36. https://doi.org/10.24394/NatSom.2020.35.29

Sano, K., Bannon, N., & Greene, M. J. (2018). Pavement Ant Workers (Tetramorium caespitum) Assess Cues Coded in Cuticular Hydrocarbons to Recognize Conspecific and Heterospecific Non-Nestmate Ants. *Journal of Insect Behavior*, *31*(2), 186–199. https://doi.org/10.1007/s10905-017-9659-4

Sisk, C. B., Shorey, H. H., Gerber, R. G., & Gaston, L. K. (1996). Semiochemicals that disrupt foraging by the Argentine ant (Hymenoptera: Formicidae): Laboratory bioassays. *Journal of Economic Entomology*, *89*(2), 381–385. https://doi.org/10.1093/jee/89.2.381

Staddon, B. W., & Everton, I. J. (1980). Haemolymph of the milkweed bug *Oncopeltus fasciatus* (Heteroptera; lygaeidae): Inorganic constituents and amino acids. *Comparative Biochemistry and Physiology Part A: Physiology*, *65*(3), 371–374. https://doi.org/10.1016/0300-9629(80)90046-8

Stadler, B., & Dixon, A. F. G. (2005). Ecology and Evolution of Aphid-Ant Interactions. *Annual Review of Ecology, Evolution, and Systematics*, *36*(1), 345–372. https://doi.org/10.1146/annurev.ecolsys.36.091704.175531

Steck, K. (2012). Just follow your nose: Homing by olfactory cues in ants. *Current Opinion in Neurobiology*, *22*(2), 231–235. https://doi.org/10.1016/j.conb.2011.10.011

Steiner, F. M., Schlick-Steiner, B. C., VanDerWal, J., Reuther, K. D., Christian, E., Stauffer, C., Suarez, A. V., Williams, S. E., & Crozier, R. H. (2008). Combined modelling of distribution and niche in invasion biology: A case study of two invasive Tetramorium ant species: Distribution and niche in invasion modelling. *Diversity and Distributions*, *14*(3), 538–545. https://doi.org/10.1111/j.1472-4642.2008.00472.x

Suckling, D. M., Peck, R. W., Stringer, L. D., Snook, K., & Banko, P. C. (2010). Trail pheromone disruption of Argentine ant trail formation and foraging. *Journal of Chemical Ecology*, *36*(1), 122–128. https://doi.org/10.1007/s10886-009-9734-1

Suckling, D. M., Stringer, L. D., & Corn, J. E. (2011). Argentine ant trail pheromone disruption is mediated by trail concentration. *Journal of Chemical Ecology*, *37*(10), 1143–1149. https://doi.org/10.1007/s10886-011-0019-0

Sunamura, E., Suzuki, S., Nishisue, K., Sakamoto, H., Otsuka, M., Utsumi, Y., Mochizuki, F., Fukumoto, T., Ishikawa, Y., Terayama, M., & Tatsuki, S. (2011). Combined use of a synthetic trail pheromone and insecticidal bait provides effective control of an invasive ant. *Pest Management Science*, *67*(10), 1230–1236. https://doi.org/10.1002/ps.2172

Tumlinson, J. H., Moser, J. C., Silverstein, R. M., Brownlee, R. G., & Ruth, J. M. (1972). A volatile trail pheromone of the leaf-cutting ant, Atta texana. *Journal of Insect Physiology*, *18*(5), 809–814. https://doi.org/10.1016/0022-1910(72)90018-2

Van Den Dool, H., & Kratz, P. Dec. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography A*, *11*, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X

Van Vorhis Key, S. E., & Baker, T. C. (1982). Trail-following responses of the Argentine ant, *Iridomyrmex humilis* (Mayr), to a synthetic trail pheromone component and analogs. *Journal of Chemical Ecology*, *8*(1), 3–14. https://doi.org/10.1007/BF00984000

Vander Meer, R. K., & Alonso, L. E. (1998). Pheromone directed behaviour in ants. In R. K. Vander Meer, M. D. Breed, K. E. Espelie, & M. L. Winston (Eds.), *Pheromone communication in social insects* (pp. 159–192).

Vander Meer, R. K., Lofgren, C. S., & Alvarez, F. M. (1990). The orientation inducer pheromone of the fire ant <i>Solenopsis invicta<i/>. *Physiological Entomology*, *15*(4), 483–488. https://doi.org/10.1111/j.1365-3032.1990.tb00537.x

Völkl, W., Woodring, J., Fischer, M., Lorenz, M. W., & Hoffmann, K. H. (1999). Ant-aphid mutualisms: The impact of honeydew production and honeydew sugar composition on ant preferences. *Oecologia*, *118*(4), 483–491.

Wagner, H. C., Arthofer, W., Seifert, B., Muster, C., Steiner, F. M., & Schlick-Steiner, B. C. (2017). Light at the end of the tunnel: Integrative taxonomy delimits cryptic species in the *Tetramorium caespitum* complex (Hymenoptera: Formicidae). *Myrmecological News*, *25*, 95–129.

Welzel, K. F., & Choe, D.-H. (2016). Development of a pheromone-assisted baiting technique for Argentine ants (Hymenoptera: Formicidae). *Journal of Economic Entomology*, *109*(3), 1303–1309. https://doi.org/10.1093/jee/tow015

Wheeler, W. M. (1927). The Occurrence of the pavement ant (*Tetramorium caespitum L.*) in Boston. *Psyche: A Journal of Entomology*, *34*, 164–165.

Zhang, Y. M., Vitone, T. R., Storer, C. G., Payton, A. C., Dunn, R. R., Hulcr, J., McDaniel, S. F., & Lucky, A. (2019). From pavement to population genomics: Characterizing a long-established non-native ant in North America through citizen science and ddRADseq. *Frontiers in Ecology and Evolution*, *7*(453). https://doi.org/10.3389/fevo.2019.00453

1.5. **Tables**

Table 1.1 List of trail pheromone components identified in *Tetramorium* and *Myrmica* ant species

Ant species	Chemical(s)	References
T. caespitum	2,5-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine	Attygalle & Morgan, 1983
T. impurum	methyl 2-hydroxy-6-methylbenzoate	Morgan & Ollett, 1987
T. meridionale	indole, methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine	Jackson et al., 1990
T. tsushimae	methyl 2-hydroxy-6-methylbenzoate	Nakamura et al., 2019
M. rubra	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. ruginodis	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. sabuleti	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. scabrinodis	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. lobicornis	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. sulcinodis	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. rugulosa	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982
M. schencki	3-ethyl-2,5-dimeythylpyrazine	Evershed et al., 1982

Chapter 2.

Identification of the trail pheromone of the pavement ant *Tetramorium immigrans* (Hymenoptera: Formicidae)*

*A near identical version of this chapter has been published: Chalissery, Jaime M., Regine Gries, Santosh K. Alamsetti, Madison J. Ardiel, and Gerhard Gries. *Journal of Chemical Ecology*, 48(3), 302-311 (2022). https://doi.org/10.1007/s10886-021-01317-3. JMC & GG conceived the study; RG & JMC ran electrophysiological recordings; SKA synthesized trail pheromones; RG prepared pheromone blends; JMC & MJA ran behavioral bioassays; JMC & MJA graphed and analyzed data; JMC & GG wrote the first draft; all authors reviewed and approved of the final draft for submission.

2.1. Abstract

Four species of *Tetramorium* pavement ants are known to guide foraging activities of nestmates via trail pheromones secreted from the poison gland of worker ants but the trail pheromone of *T. immigrans* is unknown. Our objectives were to (1) determine whether poison gland extract of *T. immigrans* workers induces trail-following behavior of nestmates, (2) identify the trail pheromone, and (3) test whether synthetic trail pheromone induces trail-following behavior of workers. In laboratory no-choice bioassays, ants followed poison-gland-extract trails farther than they followed whole-body-extract trails or solvent-control trails. Gas chromatographic-electroantennographic detection (GC-EAD) analyses of poison gland extract revealed a single candidate pheromone component (CPC) that elicited responses from worker ant antennae. The CPC mass spectrum indicated, and an authentic standard confirmed, that the CPC was methyl 2-methoxy-6-methylbenzoate (MMMB). In further laboratory no-choice bioassays, ants followed poison-gland-extract trails (tested at 1 ant equivalent) and synthetic MMMB trails (tested at 0.35 ant equivalents) equally far, indicating that MMMB is the single-component trail pheromone of *T. immigrans.* Moreover, in laboratory two-choice bioassays, ants followed MMMB trails ~21-times farther than solvent-control trails. In field settings, when *T. immigrans* colonies were offered a choice between two paper strips treated with a synthetic MMMB trail or a solvent-control trail each leading to an apple bait, the MMMB trails efficiently recruited nestmates to baits.

Keywords Myrmicinae · communication · gas chromatographic-electroantennographic detection · trail-following · field testing

2.2. **Introduction**

Except for polar regions, ants occupy every terrestrial habitat (Hölldobler & Wilson, 1990). Pavement ants thrive in urban settings, where they nest beneath, and forage along, sidewalks and driveways (Buczkowski & Richmond, 2012; Penick et al., 2015; JMC, pers. obs.). *Tetramorium immigrans* is the most common pavement ant in North America (Flucher et al., 2021; Wagner et al., 2017). It is native to the western Palaearctic and was likely introduced to North America in the 1800s (Brown, 1957; Flucher et al., 2021). It is now found in British Columbia (BC), Ontario, and Quebec (Guénard, 2017), and in both urban and agricultural environments in the U.S.A. (Helms et al., 2021; Zhang et al., 2019).

Like many other ants, *Tetramorium* colonies recruit nestmates to food sources via trail pheromones that foraging ants deposit as chemical orientation guidelines for nestmates (Beckers et al., 1990; Czaczkes et al., 2015; Morgan, 2009). To date, trail pheromones are known for four *Tetramorium* species: *T. caespitum* (3-ethyl-2,5-dimethylpyrazine, 2,5-dimethylpyrazine; Attygalle & Morgan, 1983), *T. impurum* (methyl 2-hydroxy-6-methylbenzoate; Morgan & Ollett, 1987), *T. meridionale* (indole, methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine; Jackson et al., 1990), and *T. tsushimae* (2-hydroxy-6-methylbenzoate; Nakamura et al., 2019). In all four *Tetramorium* species, it is the poison gland that produces and secretes the trail pheromone (Attygalle & Morgan, 1983; Jackson et al., 1990; Morgan & Ollett, 1987; Nakamura et al., 2019), as has been shown for more than 30 myrmicine ant species (Cerdá et al., 2014; Morgan, 2009).

Our objectives were to (1) determine whether poison gland extract of *T. immigrans* workers induces trail-following behavior of worker ants, (2) identify the trail pheromone, and (3) test whether synthetic trail pheromone induces trail-following behavior of worker ants in both laboratory and field settings.

2.3. **Methods and Materials**

2.3.1. **Experimental Insects**

Laboratory colonies of *T. immigrans* were established between July and September 2019 from natural nests located within the Iona Island Causeway (Latitude: 49°12'41" N, Longitude: 123°12'08" W; Richmond, BC, CA). From various nest entrances that were separated by at least 1 m, > 200 worker ants were collected and placed in bins (41 × 29 × 24 cm or 58 × 43 × 31 cm) that were housed in the Science Research Annex on the Burnaby campus (49°16′33″ N, 122°54′55″ W) of Simon Fraser University. The room temperature was set to 25 °C and the photoperiod to 12L:12D. The bins contained a 5–10-cm thick layer of the same substrate (sand, rocks, wood) in which field nests resided from which ants were collected. Three times per week, each nest was provisioned with food consisting of ambrosia apples as well as euthanized mealworms, *Tenebrio molitor*, and German cockroaches, *Blattella germanica*. Water was provided in a test tube (10 mL) fitted with a piece of cotton which was replaced when it became dry. Twice per week, the bins were sprayed with water to ensure adequate moisture in the nest substrate and foraging area.

2.3.2. **Preparation of Poison Gland Extract**

To obtain poison gland extract, each of 60 worker ants was cold-euthanized on dry ice and submerged in distilled water. Using fine hard-tipped forceps, the stinger – with the poison gland and the Dufour's gland attached – was gently pulled and thereby dislodged so that it floated to the water surface. The two glands were identified by reference to figures from Hölldobler & Wilson (1992) and were separated using an insect pin, forceps, and dissection scissors. The poison gland of 30 workers was placed into a vial kept on dry ice and filled with 800 µL of dichloromethane (DCM; EMD Millipore Corp., Billerica, MA, U.S.A.). Gland tissues were macerated, using a glass stirring rod and a vortex mixer (Labnet International Inc., Edison, NJ, U.S.A.), and were then filtered through a ~10-mg glass wool plug (5 × 3 mm diam.) in a glass pipette to obtain 'tissue-free' poison gland extract. Two samples of 30 glands each that had been obtained on different days were combined in a single poison gland extract, and aliquots of 1 ant equivalent (1 AE) in 25 µL of DCM were tested in trail-following experiments (see below).

2.3.3. **Preparation of Whole-body Extract**

To account for the (unlikely) possibility that the trail pheromone of *T. immingrans* is produced by a gland other the poison gland, and not knowing the location of this gland, we also obtained a 'whole-body extract' of worker ants. To this end, the severed head, thorax, and abdomen of 30 cold-euthanized ants were placed in three separate (tagma-specific) vials kept on dry ice and filled with 1 mL of DCM. After macerating body tagmata with a glass rod, syringe plunger, and a vortex mixer (Labnet International Inc., Edison, NJ, U.S.A.), each of the three samples was filtered through a glass wool plug in a glass pipette (see above) to obtain 'tissue-free' head, thorax, and abdomen extracts, respectively. After taking subsamples of these extracts for chemical analyses (not reported here), the extracts were combined in a single whole-body extract, and aliquots of 1 AE in 25 µL of DCM were tested in trail-following experiments (see below).

2.3.4. **Circular Trail-following Experiment 1 (Laboratory Setting)**

The experimental design was slightly modified from previous descriptions (Chalissery et al., 2019; Renyard et al., 2019). Briefly, to standardize visual cues during bioassays, all bioassays were run under a metal scaffold (123 × 57 × 36 cm) enclosed in black fabric, lit from above with two lights (one plant light and one daylight fluorescent light, each 48″ 32 W F32T8; Phillips, Amsterdam, NL), and fitted with a video camera (Sony HDR CX210, Sony, Tokyo, JP) to record the ants' behavior. Using a micro-syringe, a test stimulus was applied as a continuous 25-cm long trail 5 mm from the edge of a circular Whatman filter paper (90 mm diam.; Sigma-Aldrich), with the circumference marked with pencil in 1-cm intervals. This filter paper was then placed in a Pyrex glass Petri dish arena (150 mm diam.; Figure 2.1a).

Bioassay ants were isolated singly in a 1.5-mL micro-tube (Axygen™ MaxyClear Snaplock; Thermo Fisher Scientific, Waltham, MA, U.S.A.) and allowed 5 min to acclimate in the scaffold enclosure (see above). To initiate a bioassay, a micro-tube was placed in the Petri dish arena so that its exit hole was ~1 cm away from the edge of the filter paper (Figure 2.1a) and the ant was given 5 min to leave the tube. Once an ant had exited the micro-tube, her behavior was video recorded 5 min. Video footage was analyzed using VLC Media Player (Version 2.2.6) or QuickTime Player (Version 10.4) to count the number of 1-cm intervals a trail-following ant had crossed during a bioassay,

allowing us to determine the total distance she had covered as a measure of orientation in response to the test stimulus (Chalissery et al., 2019; Morgan, 2009; Renyard et al., 2019). Ants that did not exit the micro-tube within 5 min were considered non-responders and were excluded from statistical analyses. For each replicate testing a single ant, a new filter paper was used.

Experiment 1 (n = 10) tested three treatments: (1) poison gland extract (1 AE in 25 µl DCM), (2) whole-body extract (1 AE in 25 µl DCM), and (3) a solvent control (25 µl DCM). The same number of replicates was run for each treatment on any bioassay day. Between bioassays, preparative surfaces and the Petri dish arenas were cleaned with 70% ethanol and hexane, and the room was aired out for 5–10 min by opening an exterior door.

2.3.5. **Gas Chromatographic-Electroantennographic Detection (GC-EAD) and GC-Mass Spectrometric (MS) Analyses of Poison Gland Extract**

With evidence that ants follow poison-gland-extract trails significantly farther than solvent-control trails (see Results), the poison gland extract was analyzed by GC-EAD and GC-MS in splitless mode (1-2 µL injection volume), using procedures and equipment modified from previous reports (Gries et al., 2002). Briefly, the GC-EAD setup employed a Hewlett-Packard (HP) 5890 gas chromatograph (GC) fitted with a DB-5 GC column (30 m × 0.32 mm I.D., film thickness 0.25 µm; J & W Scientific, Folsom, CA, U.S.A.). Helium served as carrier gas (35 cm · s-1) with the following oven program: 100 °C for 1 min., then 20 °C · min-1 to 280 °C. The injector port and flame ionization detector (FID) were set at 260 °C. For GC-EAD recordings (N = 2), an antenna was carefully dislodged from the head of a worker ant and suspended between two glass capillary electrodes [1.0 mm outer diameter (0.58 mm inner diameter) × 100 mm; A-M Systems, Carlsborg, WA, U.S.A.] adapted to accommodate the ant antenna (~1 mm in length) and filled with saline (Staddon & Everton, 1980). Antennal responses to compounds in the column effluvium – that was directly released into a stream of medical air (250 mL/min flow) continuously passing over the electrode-suspended antenna – were amplified with a custom-built amplifier and recorded on an HP 3392A integrator. The compound in the poison gland extract that elicited a response from ant antennae (see Results) was considered a candidate pheromone component (CPC) and was

analyzed by GC-MS in full-scan electron ionization mode, using an Agilent 7890B GC coupled to a 5977A MSD (Agilent Technologies Inc., Santa Clara, CA, U.S.A.) and fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm). The injector port was set to 250 °C, the MS source to 230 °C, and the MS Quad to 150 °C. Helium was used as a carrier gas at a flow rate of 35 cm s-1 with the following temperature program: 50 °C held for 5 min, 10 °C min-1 to 280 °C (held for 10 min).

To determine the structure of the CPC, its mass spectrum and retention index (relative to aliphatic alkanes; Van Den Dool & Kratz, 1963) were compared with those of authentic standards that were synthesized in our laboratory (see below). To quantify the amount of CPC in the poison gland extract, its GC peak area was compared with that a synthetic standard prepared at 1 ng/µl.

2.3.6. **Syntheses of Candidate Pheromone Components**

Methyl 2-methoxy-6-methylbenzoate

2-Hydroxy-6-methylbenzoic acid (1 mmol, 152 mg), iodomethane (6 mmol, 850 mg) and K_2CO_3 (7.5 mmol, 1036 mg) were suspended in 5 mL of dimethylformamide (DMF) and heated to 70 °C for 16 h. After cooling to room temperature (RT), the solution was diluted in water and extracted with ethyl acetate. After drying over Na_2SO_4, the organic phase was evaporated and the resulting oil was purified by flash chromatography (silica gel, hexane/ethyl acetate 3:1), affording methyl 2-methoxy-6-methylbenzoate (126 mg, 70% yield) as a yellow oil. The nuclear magnetic resonance (NMR) spectroscopy data were in agreement with those previously reported (Baur et al., 2013).

Methyl 2-hydroxy-4,6-dimethylbenzoate

4,6-Dimethylsalicylic acid (1 mmol, 166 mg) in MeOH (5 mL) was treated at RT with 0.1 mL of sulfuric acid (95%) and 0.1 g molecular sieves (0.4 nm). After heating the reaction mixture at reflux for 72 h, it was cooled to RT, filtered, and the residue was washed with MeOH. The filtrate was evaporated, and the residue was dissolved in CH_2Cl_2, poured into crushed ice, and extracted twice with CH_2Cl_2. The organic phases were washed with water, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography (hexane/EtOAc 4:1), affording methyl 2-

hydroxy-4,6-dimethylbenzoate (72 mg, 40% yield) as a colorless solid. The NMR spectroscopy data were in agreement with those previously reported (Reim et al., 2009).

2.3.7. **Circular Trail-following Experiment 2 (Laboratory Setting)**

Following the identification of methyl 2-methoxy-6-methylbenzoate (MMMB) as the candidate trail pheromone component (see Results), experiment 2 (n = 20) compared trail-following behaviour of ants in response to (1) synthetic MMMB (2.5 ng; 0.35 AE in 25 µl DCM), (2) poison gland extract (1 AE in 25 µl DCM), and (3) a solvent control stimulus (25 µl DCM). As we assumed that ants secrete only a portion of their poison gland pheromone reservoir when marking trails, we kept the amount of synthetic MMMB, conservatively, about 3-fold lower than that of ant-produced MMMB. The protocol of experiment 2 closely followed that of experiment 1 (see above).

2.3.8. **Choice-of-trail Experiment (Laboratory Setting)**

With data revealing, in circular trail-following experiment 2, that ants follow synthetic pheromone trails significantly farther than solvent-control trails (see Results), we wanted to ascertain that they did not just follow the edge of the circular filter paper. Therefore, we adopted the design of a ‘choice-of-trail’ experiment (n = 18) previously described (Renyard et al. 2019). Briefly, a plexiglass arena (64 × 44 × 10 cm) was fitted with a sheet of white printer paper (22 × 28 cm) marked with two pencil lines in V-shape (45° angle), with each line divided in 25 1-cm intervals (Figure 2.1b). For each replicate, the treatment stimulus (synthetic MMMB (2.5 ng) at 0.35 AE in 25 µL DCM) and the control stimulus (25 µL DCM) were randomly assigned to one of the two lines and applied with a micro-syringe. To initiate a bioassay replicate, the micro-tube containing a single ant was placed into the arena such that the exit hole of the tube pointed towards the intersection of the “V”. For each ant, we recorded the distance she walked along either line. Ants that did not exit the micro-tube within 5 min were considered non-responders and were excluded from statistical analyses. For each replicate testing a single ant, a new filter paper was used, and all surfaces were cleaned with 70% ethanol and hexane.

2.3.9. **Choice-of-trail Experiment (Field Setting)**

To determine whether synthetic trail pheromone affects nestmate recruitment in field settings, a choice-of-trail experiment was run in Surrey (49°10'20"N 122°42'09"W), BC, CA in June 2020, and in Burnaby (49°12'29"N 122°59'22"W), BC, CA in June 2021 (n = 11). Ant colonies with more than 20 ants near the nest entrance at the time of assessment, or with more than 50 foragers recruited within 30 min to an apple-slice bait placed 25 cm from the nest entrance, were deemed to have high foraging activity, whereas all others were considered colonies of low foraging activity.

For each replicate, two strips of filter paper (25 × 2 cm each) were placed at 0º and 180º as close as possible to the entrance of a *T. immigrans* nest (Figure 2.1c), taking great care not to disrupt debris near the entrance. To minimize the likelihood of recruiting the same foragers in various replicates, only nests with entrances separated by at least 0.5 m were selected for testing. To prevent wind from moving strips, they were weighed down with several nails placed along the edges (but not center) of strips. The distant end of each strip was baited with a small cylinder (~ 0.5 × 2 cm) of an ambrosia apple presented on a piece of paper (4.5 × 4.5 cm) to prevent ant access from below. After placing apple baits, the treatment stimulus (synthetic MMMB (2.5 ng) at 0.35 AE in 25 µL DCM) and the control stimulus (25 µL DCM) were randomly assigned to one of the two strips and applied with a micro syringe. To determine whether synthetic MMMB expedites recruitment of nestmates to baits, photos of ants at food baits were taken at 5-min intervals for 50 min.

2.3.10. **Statistical Analyses**

We analyzed all data with R 3.6.3 using packages “multcomp”, “plotrix”, “plyr”, “nlme”, and “emmeans”. Data (distance followed) of circular trail-following experiments were analyzed using a generalized linear model (GLM; quasi-Poisson distribution). The mean and standard error for distance followed by ants in response to test stimuli were analyzed by an analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) tests. Data (proportional distance walked by ants on pheromone trails or solvent-control trails) of choice-of-trail experiments (laboratory setting) were compared against a theoretical 50:50 distribution using a $\chi 2$-test. The mean and standard error for distance walked by ants in response to test stimuli were analyzed by a

paired t-test. For the choice-of-trail experiments (field setting), we analyzed the number of foraging ants on food baits at the end of either a pheromone trail or solvent-control trail over 10 5-min time points using a linear mixed-effects model. The mean and standard error for number of foraging ants in response to treatment and time point (with colony and location as random variables) were analyzed by a repeated measures analysis of variance (ANOVA).

2.4. **Results**

2.4.1. **Circular Trail-following Experiment 1 (Laboratory Setting)**

The distances worker ants followed trails of (*i*) poison gland extract, (*ii*) whole-body extract or (*iii*) a solvent control significantly differed (ANOVA, $F_{(2, 27)} = 11.6$, $P < 0.001$; Figure 2.2a). Ants followed poison-gland-extract trails farther than whole-body-extract trails (z = 3.24, $P = 0.00325$) or solvent-control trails (z = 3.52, $P = 0.00177$) but followed whole-body-extract trails and solvent-control trails equally far (z = 0.669, $P = 0.777$).

2.4.2. **GC-EAD and GC-MS Analyses of Poison Gland Extract**

GC-EAD analyses of *T. immigrans* poison gland extract revealed a single candidate pheromone component (marked as 'CPC' in Figure 2.3a) that consistently elicited responses from worker ant antennae. The mass spectrum of CPC (Figure 2.3b) indicated a nominal mass of 180 u, and fragmentation ions indicative of an aromatic ring (*m/z* 77) and a methyl branch on the ring (*m/z* 91). Overall, the CPC mass spectrum resembled that of methyl 2-hydroxy-6-methylbenzoate (MW 166), but the CPC had one additional carbon. We hypothesized that the CPC has a second methyl branch on the aromatic ring or a methoxy group, instead a hydroxyl group, at C2. Keeping the methyl branch conservatively at C6, we first synthesized methyl 2-hydroxy-4,6-dimethylbenzoate which eluted later than the CPC. We then prepared methyl 2-methoxy-6-methylbenzoate (MMMB) which had a mass spectrum and a GC retention index consistent with those of the CPC. With this information in hand, we then determined that the poison gland of an individual ant contained about 7 ng of MMMB.

2.4.3. **Circular Trail-following Experiment 2 (Laboratory Setting)**

The distances worker ants followed trails of (*i*) poison gland extract, (*ii*) synthetic MMMB, and (*iii*) a solvent control significantly differed (ANOVA, $F_{(2, 57)} = 19.8$, $P < 0.001$; Figure 2.2b). Ants followed poison-gland-extract trails and synthetic MMMB trails equally far ($z = -0.647$, $P = 0.787$) but followed trails of either stimulus farther than solvent-control trails (poison gland extract: $z = 4.80$, $P < 0.001$; synthetic MMMB: $z = 4.42$, $P < 0.001$).

2.4.4. **Choice-of-trail Experiment (Laboratory Setting)**

When presented with a choice between two pencil lines drawn in V-shape and treated with either synthetic MMMB or a solvent control, the proportion of distance that ants followed either line differed from a theoretical 50:50 distribution ($\chi^2 = 16.8$, df = 1, $P < 0.001$). Ants followed along the pheromone trail ~21-times farther than the solvent-control trail (Paired *t*-test: $t = -8.70$, df = 17, $P < 0.001$; Figure 2.4).

2.4.5. **Choice-of-trail Experiment (Field Setting)**

When *T. immigrans* colonies with low and high foraging activities of ants (random variable) were offered a choice between two paper strips placed at 0º and 180º from a nest entrance and treated with either a synthetic MMMB trail or a solvent-control trail each leading to an apple bait (Figure 3.1c), there was a significant effect of treatment (pheromone *vs* control) and time point (5–50 min in 5-min intervals) on the number of ants recruited to the apple bait (repeated measures ANOVA: treatment, $F_{(1,17)} = 48.4$, $P < 0.001$; time point, $F_{(9,189)} = 14.4$, $P < 0.001$; Figure 2.5).

2.5. **Discussion**

Our data support the conclusion that (1) methyl 2-methoxy-6-methylbenzoate (MMMB) is the major and possibly only component of the *T. immigrans* trail pheromone, (2) MMMB originates from the poison gland, and (3) synthetic MMMB deployed in field settings expedites recruitment of nestmates to food sources.

Drawing on the literature that trail pheromones of myrmicine ants often originate from the poison gland (Cerdá et al., 2014; Morgan, 2009), we excised the poison gland

from the abdomen of ants, extracted the glands in DCM, and bioassayed poison gland extract for trail-following behavior of ants (Figure 2.2a). To address the remote possibility that the trail pheromone of *T. immigrans* may originate from a gland other than the poison gland, but not knowing the location of this gland, we also prepared extracts of the abdomen, thorax and head, and combined aliquots of these three separate extracts in a whole-body extract for trail-following bioassays. As predicted, ants followed poison-gland-extract trails tested at 1 ant equivalent significantly farther than they followed solvent-control trails (Figure 2.2a). Unexpectedly however, ants followed whole-body-extract trails (which contained the trail pheromone) not farther than they followed solvent-control trails (Figure 2.2a). As poison gland extract and (recombined) whole-body extract contained trail pheromone at similar amounts, it follows that the whole-body extract likely contained other pheromones that function in diverse contexts, and thus may have presented a “mixed” message to ants, prompting no trail-following response. For example, the whole-body extract could have contained an alarm pheromone that may originate from an exocrine gland in the head (Blum, 1969; Pasteels et al., 1980). Analyzing head, thorax and abdomen extracts separately by GC-EAD and GC-MS in a future study, and testing synthetic compounds in behavioral bioassays, should reveal the pheromone(s) that interfered with trail-following behavior by *T. immigrans* ants when they were presented in a whole-body extract.

With convincing evidence that the trail pheromone of *T. immigrans* originates from the poison gland (Figure 2.2), we analyzed poison gland extract by GC-EAD and GC-MS. The single compound that consistently elicited responses from ant antennae (Figure 2.3a) had a mass spectrum which resembled that of methyl 2-hydroxy-6-methylbenzoate [the trail pheromone of *T. impurum* (Morgan et al., 1990)] but indicated one additional carbon (Figure 2.3b). Of the two most likely structures, methyl 2-hydroxy-4,6-dimethylbenzoate and methyl 2-methoxy-6-methylbenzoate (MMMB), that we prepared and analyzed in comparison to the natural product, only the latter had spectrometric and chromatographic characteristics entirely consistent with the ant-produced compound.

Based on this result, we prepared synthetic MMMB for circular trail-following bioassays (Figure 2.1A), testing it at 0.35 ant equivalents. Even at this low dose, ants followed MMMB trails for about the same distance as they followed poison-gland-extract trails tested at 1 ant equivalent (Figure 2.2b). These bioassay data, coupled with

electrophysiological data showing that ant antennae sense only MMMB as a single compound in poison gland extracts (Figure 2.3a), support the conclusion that MMMB is the major and possibly only component of the *T. immigrans* trail pheromone. MMMB is also a trace component in the poison gland of *T. impurum* and may enhance the effect of the major pheromone component methyl 2-hydroxy-6-methylbenzoate (Morgan et al., 1990), but no quantitative data on component interactions have been reported.

To address the possibility that *T. immigrans* ants followed MMMB trails, in part, because these trails were presented along the edge of a circular filter paper which may have served as a physical guide, we tested synthetic MMMB also in a choice-of-trail experiment, as previously described for trail pheromone testing with western carpenter ants, *Camponotus modoc* (Renyard et al., 2019). In this type of bioassay, single ants exit a holding tube and face a choice between two trails presented in V-shape without a physical edge in the center of plain paper (Figure 2.1b). When tested in this choice-of-trail bioassay, worker ants of *T. immigrans* followed synthetic MMMB trails ~21-times farther than they followed solvent-control trails, indicating that a physical guide, such as the filter paper edge in circular trail-following bioassays, is not needed for trail-following responses of *T. immigrans* worker ants. In contrast, the amount of trail pheromone deployed could have a strong effect on trail-following behavior, but it is not necessarily the largest amount that elicits the strongest trail-following response (Renyard et al., 2019; and references therein). The optimal dose of MMMB for trail-following of *T. immigrans* workers has yet to be determined.

With convincing data that synthetic MMMB elicits trail-following behavior by *T. immigrans* in laboratory bioassays (Figures. 2.2, 2.4), there was incentive to test the effect of synthetic MMMB also in field settings. To this end, we ran a choice-of-trail experiment with multiple *T. immigrans* nests. For each nest, we placed two paper strips at 0° and 180° from the nest entrance (Figure 2.1c), baited the distant end of each strip with a piece of apple, and applied synthetic MMMB or a solvent-control stimulus to each strip. As trail pheromones serve to recruit nestmates to food sources (Beckers et al., 1990; Czaczkes et al., 2015; Morgan, 2009), we recorded the number of nestmates recruited to apple baits, rather than distance followed, as the response criterion. Our field data show that trails of synthetic MMMB greatly expedite recruitment of nestmates to apple baits (Figure 2.5). The recruitment effect of synthetic MMMB was clearly apparent in settings of both low and high ant foraging activity but the effect was

particularly pronounced in low ant activity settings (Figure 2.5a). There, pheromone trails prompted expeditious recruitment of nestmates to apple baits, and thus led to their efficient exploitation. While workers in high ant activity settings quickly located the pheromone trails and apple baits, the number of ants recruited to baits plateaued early (Figure 2.5b), thereby preventing trail and resource 'overcrowding'. Both data sets reflect an optimal foraging strategy, as reported for many ants (Czaczkes et al., 2015; Detrain & Deneubourg, 2008).

Surprisingly, synthetic trail pheromone has been field-tested for trail-following behavior of ants in only one other study, dating back 40 years (Tumlinson et al., 1972). In this study, medium and large workers of the leaf-cutting ant, *Atta texana*, readily followed the trail pheromone component methyl 4-methylpyrrole-2-carboxylate when applied as a 2.7-pg/cm trail on a cardboard strip placed across an "erased" section of the natural pheromone trail. According to this study, the ants also responded to a synthetic pheromone trail that was dribbled on sand at a 45° angle from the natural trail.

More recent field studies have tested synthetic trail pheromone (*Z*-9-hexadecenal) of Argentine ants as a means of enhancing the attractiveness and consumption of lethal baits (Greenberg & Klotz, 2000; Welzel & Choe, 2016) or to disrupt foraging activities of ants (Nishisue et al., 2010, 2020; Sisk et al., 1996; Suckling et al., 2010, 2011; Sunamura et al., 2011). For example, permeating the environment with synthetic pheromone suppressed foraging activities of Argentine ants in pheromone treatment plots (Nishisue et al., 2020). There is no field study, however, that explored the ability of synthetic trail pheromone trails to guide foraging Argentine ants, or other ants, to a (lethal) food bait. The reasons for this are not known but may include (*i*) incomplete knowledge of the entire trail pheromone blend, (*ii*) lack of effective trail pheromone formulations, (*iii*) expectations that ants eventually find lethal food baits irrespective of trail pheromone deployment, and (*iv*) no prospect of commercializing synthetic trail pheromone lures for single species of ants. While further field studies are needed, our field data imply that the deployment of synthetic trail pheromone has potential to expeditiously guide ants to lethal food baits. This tactic may prove effective at early stages of new ant invasions when population densities are still low or immediately following population knockdowns through various control measures.

In conclusion, we show that methyl 2-methoxy-6-methylbenzoate (MMMB) is the major and possibly only component of the *T. immigrans* trail pheromone, and that synthetic MMMB deployed in field settings expedites the recruitment of nestmates to baits. Our field data provide incentive to the pest control industry to formulate synthetic trail pheromones as a means to promote ant foraging on lethal baits that quickly lead to the demise of nests (e.g., Hoefele et al., 2021). This type of control tactic, while it may be deemed not necessary for nuisance pests such as *T. immigrans*, might help control major invasive ant pests such as the red imported fire ant, *Solenopsis invicta*, and the tawny crazy ant, *Nylanderia fulva*. Commercial viability of this tactic may be achieved by combining pheromones of multiple species in a single formulation, with potential synergism and no antagonism between pheromone components (Chalissery et al., 2019).

2.6. **Acknowledgements**

We thank two anonymous reviewers for constructive comments, Devon Rai and his family for permission to run replicates of the field experiment on their property, Adam Blake and Asim Renyard for advice on statistical analyses and graphical illustrations, Asim Renyard and Sean McCann for help locating and identifying the ants that were collected for laboratory colonies, April Lin, Jasper Li, Kai Zhang, Kelvin Lau, Kristy Lok, Saif Nayani, and Srishti Kumar for help with ant care, and Sharon Oliver for some word processing and comments.

2.7. **Funding**

This research was supported by a MPM Graduate Entrance Scholarship, a Graduate Fellowship, the Dr. H.R. MacCarthy Graduate Bursary from Simon Fraser University, and the Mildred Wells Scholarship from the BC Council of Garden Clubs, to JMC. The research was further supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) -Industrial Research Chair to GG with BASF Canada Ltd. and Scotts Canada Inc. as the industrial sponsors.

2.8. **References**

Attygalle AB, Morgan ED (1983) Identification of trail pheromone of the ant *Tetramorium caespitum* L. (Hymenoptera: Myrmicinae). Naturwissenschaften 70:364–365. https://doi.org/10.1007/BF00990315

Beckers R, Deneubourg JL, Goss S, Pasteels JM (1990) Collective decision making through food recruitment. Insectes Sociaux 37:258–267. https://doi.org/10.1007/BF02224053

Blum MS (1969) Alarm pheromones. Annu Rev Entomol 14:57–80. https://doi.org/10.1146/annurev.en.14.010169.000421

Brown WLJ (1957) Is the ant genus *Tetramorium* native in North America? Breviora 72:1–8

Buczkowski G, Richmond DS (2012) The effect of urbanization on ant abundance and diversity: A temporal examination of factors affecting biodiversity. PLoS ONE 7:e41729. https://doi.org/10.1371/journal.pone.0041729

Cerdá X, van Oudenhove L, Bernstein C, Boulay RR (2014) A list of and some comments about the trail pheromones of ants. Nat Prod Commun 9:1934578X1400900. https://doi.org/10.1177/1934578X1400900813

Chalissery JM, Renyard A, Gries R, Hoefele D, Alamsetti SK, Gries G (2019) Ants sense, and follow, trail pheromones of ant community members. Insects 10:383. https://doi.org/10.3390/insects10110383

Chan KH, Guénard B (2020) Ecological and socio-economic impacts of the red import fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), on urban agricultural ecosystems. Urban Ecosyst 23:1–12. https://doi.org/10.1007/s11252-019-00893-3

Czaczkes TJ, Grüter C, Ratnieks FLW (2015) Trail pheromones: An integrative view of their role in social insect colony organization. Annu Rev Entomol 60:581–599. https://doi.org/10.1146/annurev-ento-010814-020627

Detrain C, Deneubourg J-L (2008) Collective decision-making and foraging patterns in ants and honeybees. Adv. Insect Physiol 35:123–173

Flucher SM, Krapf P, Arthofer W, Suarez AV, Crozier RH, Steiner FM, Schlick-Steiner BC (2021) Effect of social structure and introduction history on genetic diversity and differentiation. Mol Ecol mec.15911. https://doi.org/10.1111/mec.15911

Greenberg L, Klotz JH (2000) Argentine ant (Hymenoptera: Formicidae) trail pheromone enhances consumption of liquid sucrose solution. J Econ Entomol 93:119–122. https://doi.org/10.1603/0022-0493-93.1.119

Gries R, Khaskin G, Gries G, Bennett RG, King GGS, Morewood PS, Slessor KN, Morewood D (2002) (*Z*,*Z*)-4,7-Tridecadien-(*S*)-2-yl acetate: Sex pheromone of Douglas-fir cone gall midge, *Contarinia oregonensis*. J Chem Ecol 28:2283–2297. https://doi.org/10.1023/A:1021005517389

Gruber MAM, Janssen-May S, Santoro D, Cooling C, Wylie R (2021) Predicting socio-economic and biodiversity impacts of invasive species: red imported fire ant in the developing western Pacific. Ecol Manag Restor 22:89–99. https://doi.org/10.1111/emr.12457

Guénard B (2017) The global ant biodiversity informatics (GABI) database: synthesizing data on the geographic distribution of ant species (Hymenoptera: Formicidae). Myrmecol News 24:83–89. https://doi.org/10.25849/myrmecol.news_024:083

Helms JA, Roeder KA, Ijelu SE, Ratcliff I, Haddad NM (2021) Bioenergy landscapes drive trophic shifts in generalist ants. J Anim Ecol 90:738–750. https://doi.org/10.1111/1365-2656.13407 Hölldobler B, Wilson EO (1992) The ants. The Belknap Press of Harvard University Press

Jackson BD, Keegans SJ, Morgan ED, et al (1990) Trail pheromone of the ant *Tetramorium meridionale*. Naturwissenschaften 77:294–296

Morgan ED (2009) Trail pheromones of ants. Physiol Entomol 34:1–17. https://doi.org/10.1111/j.1365-3032.2008.00658.x

Morgan ED, Jackson BD, Ollett DG, Sales GW (1990) Trail pheromone of the ant *Tetramorium impurum* and model compounds: Structure-activity comparisons. J Chem Ecol 16:3493–3510. https://doi.org/10.1007/BF00982113

Morgan ED, Ollett DG (1987) Methyl 6-methylsalicylate, trail pheromone of the ant *Tetramorium impurum*. Naturwissenschaften 74:596–597. https://doi.org/10.1007/BF00368520

Nakamura T, Harada K, Akino T (2019) Identification of methyl 6-methylsalicylate as the trail pheromone of the Japanese pavement ant *Tetramorium tsushimae* (Hymenoptera: Formicidae). Appl Entomol Zool 54:297–305. https://doi.org/10.1007/s13355-019-00626-0

Naumann K, Sá MMD, Lewis E, Noronha R (2017) Supercolonies of the invasive ant, *Myrmica rubra* (Hymenoptera: Formicidae) in British Columbia, Canada. J Entomol Soc BC, 114: 56–64

Nishisue K, Koyama S, Satoh T (2020) Identification of the Argentine ant *Linepithema humile* (Hymenoptera: Formicidae) using an artificially synthesized trail pheromone and its effects on native Japanese ants. Appl Entomol Zool 55:141–147. https://doi.org/10.1007/s13355-019-00663-9

Nishisue K, Sunamura E, Tanaka Y, Sakamoto H, Suzuki S, Fukumoto T, Terayama M, Tatsuki S (2010) Long-term field trial to control the invasive Argentine ant (Hymenoptera: Formicidae) with synthetic trail pheromone. J Econ Entomol 103: 1784–1789. https://doi.org/10.1603/EC10008

Pasteels JM, Verhaeghe JC, Braekman JC, Daloze D, Tursch B (1980) Caste-dependent pheromones in the head of the ant *Tetramorium caespitum*. J Chem Ecol 6:467–472. https://doi.org/10.1007/BF01402923

Penick CA, Savage AM, Dunn RR (2015) Stable isotopes reveal links between human food inputs and urban ant diets. Proc R Soc B Biol Sci 282:20142608. https://doi.org/10.1098/rspb.2014.2608

Renyard A, Alamsetti SK, Gries R, Munoz A, Gries G (2019) Identification of the trail pheromone of the carpenter ant *Camponotus modoc*. J Chem Ecol 45:901–913. https://doi.org/10.1007/s10886-019-01114-z

Sisk CB, Shorey HH, Gerber RG, Gaston LK (1996) Semiochemicals that disrupt foraging by the Argentine ant (Hymenoptera: Formicidae): Laboratory bioassays. J Econ Entomol 89:381–385. https://doi.org/10.1093/jee/89.2.381

Staddon BW, Everton IJ (1980) Haemolymph of the milkweed bug *Oncopeltus fasciatus* (Heteroptera; lygaeidae): Inorganic constituents and amino acids. Comp Biochem Physiol A Physiol 65:371–374. https://doi.org/10.1016/0300-9629(80)90046-8

Suckling DM, Peck RW, Stringer LD, Snook K, Banko PC (2010) Trail pheromone disruption of Argentine ant trail formation and foraging. J Chem Ecol 36:122–128. https://doi.org/10.1007/s10886-009-9734-1

Suckling DM, Stringer LD, Corn JE (2011) Argentine ant trail pheromone disruption is mediated by trail concentration. J Chem Ecol 37:1143–1149. https://doi.org/10.1007/s10886-011-0019-0

Sunamura E, Suzuki S, Nishisue K, Sakamoto H, Otsuka M, Utsumi Y, Mochizuki F, Fukumoto T, Ishikawa Y, Terayama M, Tatsuki S (2011) Combined use of a synthetic trail pheromone and insecticidal bait provides effective control of an invasive ant. Pest Manag Sci 67:1230–1236. https://doi.org/10.1002/ps.2172

Tumlinson JH, Moser JC, Silverstein RM, Moser JC, Brownlee RG, Ruth JM (1972) A volatile trail pheromone of the leaf-cutting ant, Atta texana. J Insect Physiol 18:809–814. https://doi.org/10.1016/0022-1910(72)90018-2

Van Den Dool H, Kratz P (1963) A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. J Chromatogr A 11:463–471. https://doi.org/10.1016/S0021-9673(01)80947-X

Wagner HC, Arthofer W, Seifert B, Muster C, Steiner FM, Schlick-Steiner BC (2017) Light at the end of the tunnel: Integrative taxonomy delimits cryptic species in the *Tetramorium caespitum* complex (Hymenoptera: Formicidae). Myrmecol News 25:95–129

Wang Z, Moshman L, Kraus E, Wilson BE, Acharya N, Diaz R (2016) A Review of the tawny crazy ant, *Nylanderia fulva*, an emergent ant invader in the southern United States: Is biological control feasible management option? Insects 7:77. https://doi.org/10.3390/insects7040077

Welzel KF, Choe D-H (2016) Development of a pheromone-assisted baiting technique for Argentine ants (Hymenoptera: Formicidae). J Econ Entomol 109:1303–1309. https://doi.org/10.1093/jee/tow015

Zhang YM, Vitone TR, Storer CG, Payton AC, Dunn RR, Hulcr J, McDaniel SF, Lucky A (2019) From pavement to population genomics: Characterizing a long-established non-native ant in North America through citizen science and ddRADseq. Front Ecol Evol 7. https://doi.org/10.3389/fevo.2019.00453

2.9. **Figures**

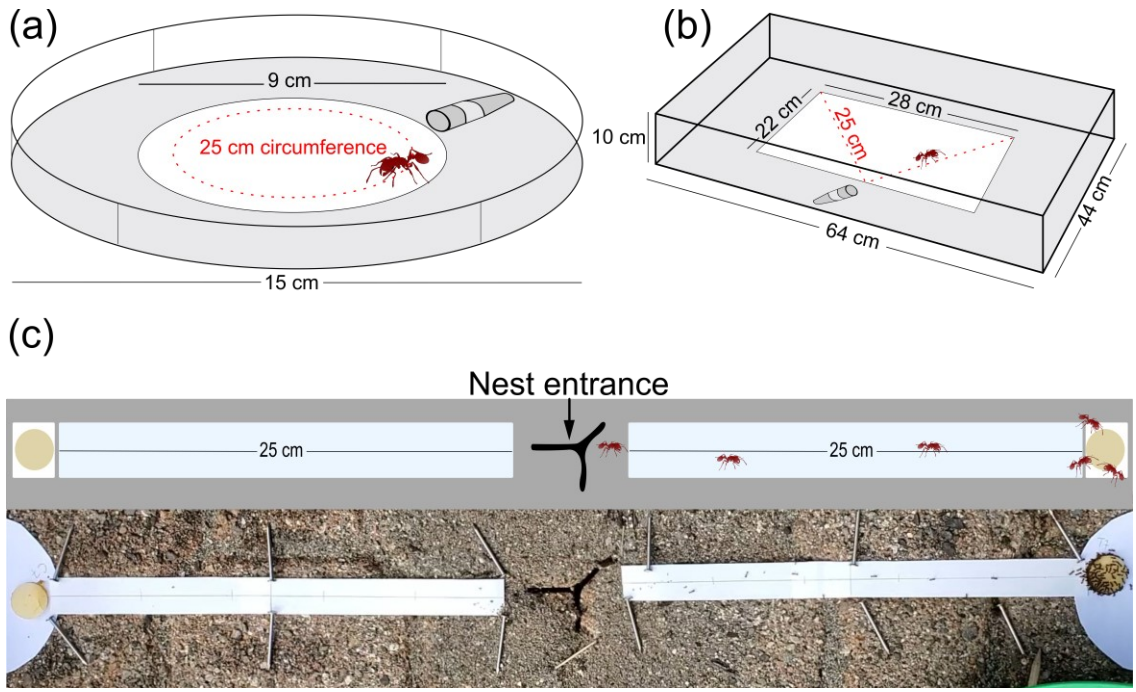


Figure 2.1 Graphic and photographic illustrations of experimental designs (drawings not to scale). (a) Design components of circular trail-following experiments, consisting of (*i*) a Pyrex petri dish arena fitted with a circular filter paper marked in 1-cm intervals along the edge; (*ii*) a holding tube from which a single ant enters the arena; and (*iii*) a 25-cm long stimulus trail (dotted line). (b) Design components of the choice-of-trail laboratory experiment, consisting of (*i*) a Plexiglass arena fitted with a sheet of paper marked with pencil lines in V-shape (dotted lines), with each line being divided in 1-cm intervals and treated with a test stimulus; and (*ii*) a holding tube from which a single ant enters the arena. (c) Design components of the choice-of-trail field experiment, consisting of (*i*) two paper strips placed at 0° and 180° from the nest entrance (denoted by arrow) and treated with a test stimulus; (*ii*) nails securing the strips to the ground; and (*iii*) an apple bait at the distant end of strips. Note the number of ants on the apple bait with a pheromone trail leading to it

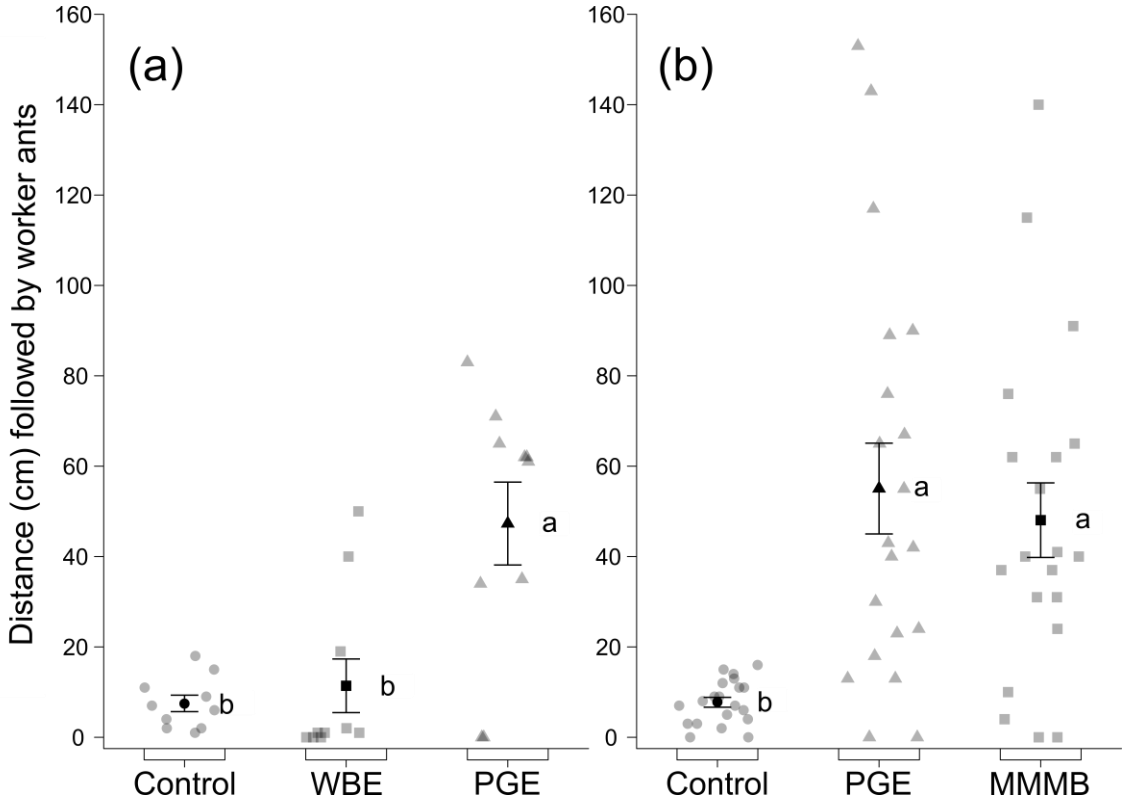


Figure 2.2 Distances that worker ants of *Tetramorium immigrans* followed trails in circular trail-following experiments (design in Figure 2.1a). Grey and black symbols show the distance that each ant, and 10 ants (a) or 20 ants (b) on average (mean ± standard error), followed trails. Test stimuli consisted of (a) a solvent control (circles), whole-body extract (WBE, 1 ant equivalent; squares) and poison gland extract (PGE, 1 ant equivalent; triangles) of *T. immigrans* workers, or (b) a solvent control (circles), poison gland extract (PGE, 1 ant equivalent; triangles) of *T. immigrans* workers, and synthetic methyl 2-methoxy-6-methylbenzoate (MMMB (2.5 ng), 0.35 ant equivalents; squares). In each subpanel, means associated with different letters are statistically different (Tukey's HSD test, $P < 0.01$)

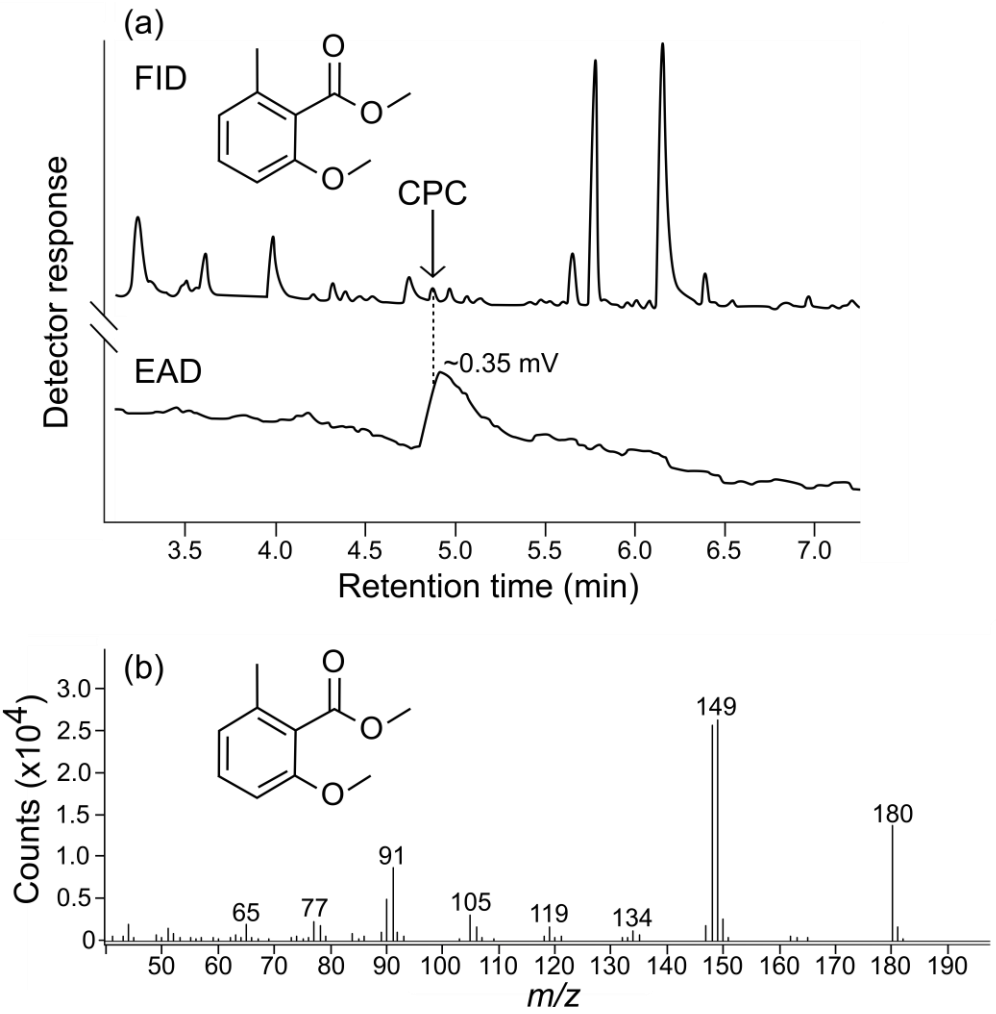


Figure 2.3 (a) Representative recording (one of two replicates) of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: antenna of a *Tetramorium immigrans* worker ant) to compounds present in poison gland extract of T. immigrans worker ants. CPC denotes methyl 2-methoxy-6-methylbenzoate; the first large peak (eluting between 5.5 and 6.0 min) was pentadecane, the second large peak (eluting between 6.0 and 6.5 min) is unknown. (b) Mass spectrum of methyl 2-methoxy-6-methylbenzoate

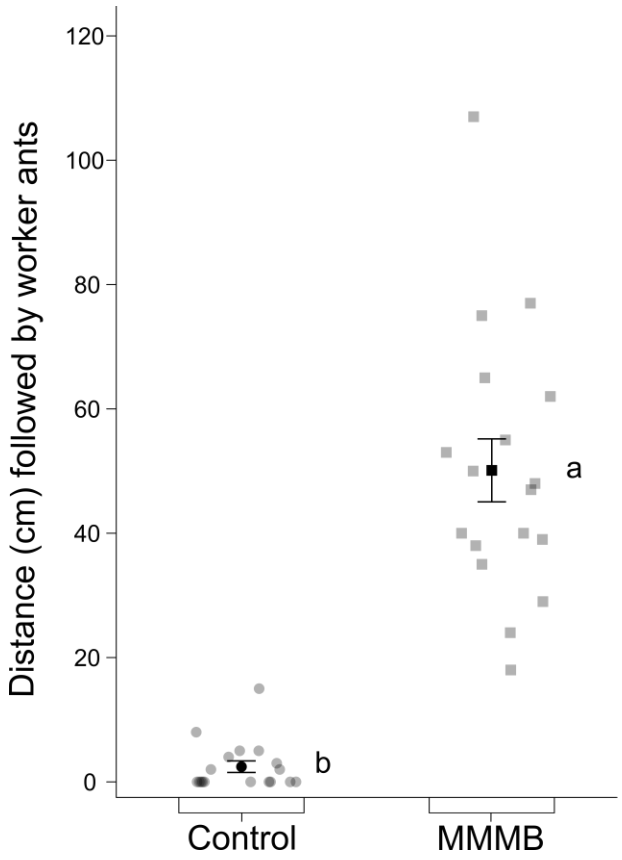


Figure 2.4 Distances worker ants of *Tetramorium immigrans* followed V-shaped trails in a choice-of-trail laboratory experiment (design in Figure 2.1b). Grey and black symbols show the distance that each ant and 18 ants on average (mean ± standard errors), followed trails. Test stimuli consisted of a solvent control (circles) and synthetic methyl 2-methoxy-6-methylbenzoate (MMMB (2.5 ng), 0.35 ant equivalents; squares) of *T. immigrans* workers. Means associated with different letters are statistically different (Tukey's HSD test, P < 0.01)

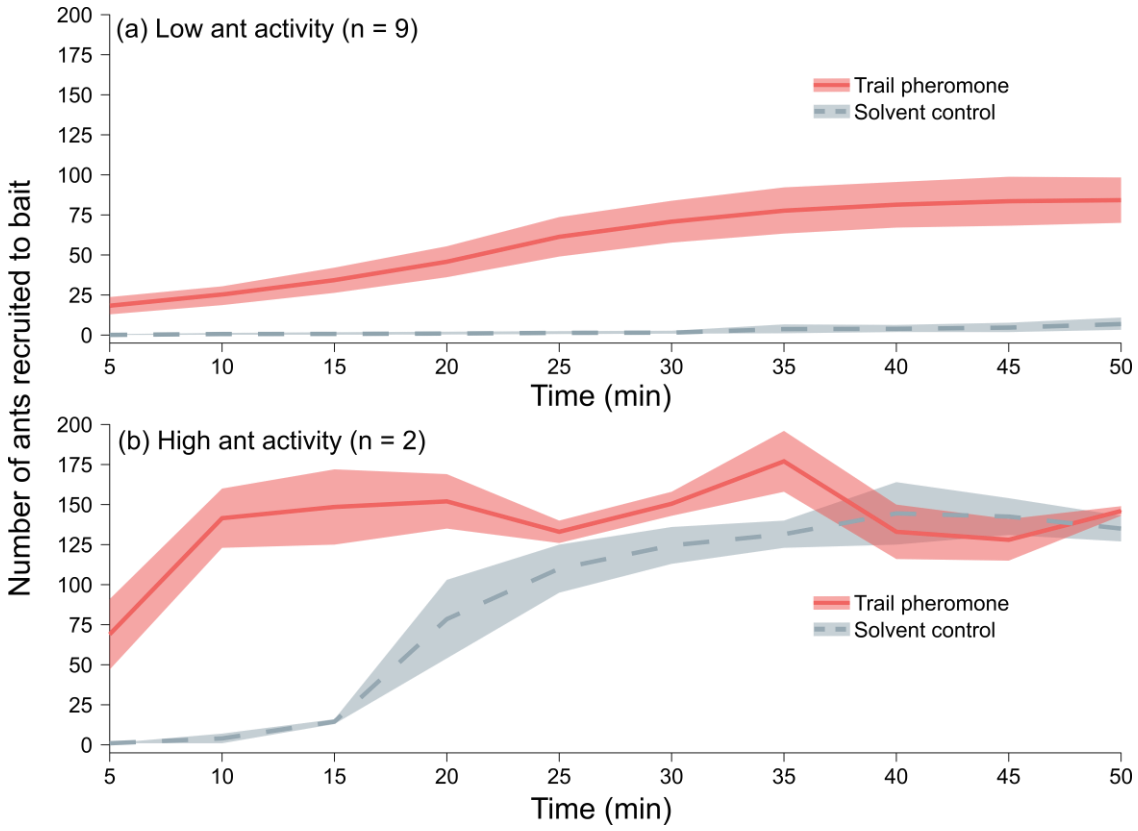


Figure 2.5 Mean numbers (solid and dotted lines; standard errors = shaded areas around lines) of *Tetramorium immigrans* worker ants recruited to apple baits when offered a choice between two paper strips placed at 0° and 180° from a nest entrance and treated with either a synthetic pheromone trail (methyl 2-methoxy-6-methylbenzoate (2.5 ng); 0.35 ant equivalents) or a solvent-control trail (design in Figure 2.1c). Analyses of all replicates (with activity level as a random variable) revealed a significant effect of treatment (pheromone vs control) and time point on the number of ants recruited to apple baits (repeated measures ANOVA: treatment, $P < 0.001$; time point, $P < 0.001$)

Note: Pronounced effect of the trail pheromone on ant recruitment in settings of low ant foraging activity

Chapter 3.

Myrmica rubra foragers produce a 3-component trail pheromone that nestmates follow in groups

3.1. Abstract

In many ant species, successful foragers deposit trail pheromone that guides nestmates to food sources. Single workers of the European fire ant, *Myrmica rubra*, reportedly do not respond to their own trail pheromone (3-ethyl-2,5-dimethylpyrazine; 'EDP') nor to a trail pheromone blend composed of EDP and trail pheromones of heterospecific ant community members of *M. rubra*. Here we tested the hypotheses that (1) the *M. rubra* trail pheromone comprises more components than EDP, (2) *M. rubra* workers respond to trail pheromone only in groups, (3) groups of *M. rubra* workers follow trail pheromones of heterospecific ant community members, and (4) synthetic trail pheromone expedites recruitment of foraging *M. rubra* ants in field settings. Coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of *M. rubra* abdominal extracts revealed three components that elicited responses from worker ant antennae. GC-mass spectrometry (MS) indicated, and GC-MS analyses of authentic standards confirmed, that they were EDP, (*Z*,*E*)-α-farnesene [(3*Z*,6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene] and (*Z*,*E*)-α-homofarnesene [(3*Z*,6*E*)-7-ethyl-3,11-dimethyldodeca-1,3,6,10-tetraene]. A synthetic blend of these three components proved superior to EDP in prompting (*i*) sustained trail-following behavior in laboratory bioassays, and (*ii*) relatively faster recruitment of foraging ants to apple baits in a field experiment. Only 5-ant groups of *M. rubra* workers, but not single workers, responded to trails of synthetic pheromone. Whether trail pheromones of heterospecific ant community members interfere with optimal trail-following behavior of *M. rubra* workers in response to their own (*sensu* conspecific) trail pheromone is still not clear.

Keywords Myrmicinae · communication · gas chromatographic-electroantennographic detection · trail-following · field testing

3.2. **Introduction**

The communication system of ant colonies is highly complex and entails information flow between queens, workers, and brood (Vander Meer & Alonso, 1998). The mode of information transmission is based mainly on chemical signals (pheromones) but vibratory and tactile signals also play a role (Hölldobler & Wilson, 1990; Keeling et al., 2004). Information conveyance among worker ants is critically important in the context of foraging. In many ant species, particularly those that form large colonies, trail pheromones help coordinate foraging efforts (Beckers et al., 1990; Billen & Morgan, 1998; Planqué et al., 2010). When a foraging ant scout has located and fed on a food source, she returns to the nest and deposits trail pheromone along the route. This trail pheromone then becomes a guide to nestmates who – on their return trip to the nest – may also deposit pheromone and thus reinforce the information (Czaczkes et al., 2015; Lehue & Detrain, 2020; Morgan, 2009). Trail pheromone-guided foraging activities may be coupled with physical modes of recruitment, where one ant leads a single nestmate (tandem running) or a group of nestmates to a resource (Planqué et al., 2010).

In their invaded North American range, European fire ants, *Myrmica rubra* (Linnaeus), are described as prolific scavengers that outcompete and aggressively displace native ant species (Garnas et al., 2014; Naumann & Higgins, 2015). Nuptial flights have rarely been observed, suggesting that introductions and long-range 'dispersal' of *M. rubra* take place through movement of ant-infested soil (Hicks, 2012). Within infested areas, short-range dispersal occurs through 'budding', a process where queens that have mated with a nestmate leave the original colony together with a cohort of workers and found a new colony nearby. Budding leads to the formation of 'super-colonies' comprising multiple nests, with near genetically identical nestmates that collaborate during foraging and defense of resources (Hicks & Marshall, 2018; Naumann et al., 2017). With no competition between colonies, and with no long-range dispersal, budding may lead to rapid expansions of *M. rubra* populations in infested locations, thus enabling *M. rubra* to become the dominant ant species in its invaded habitat and to monopolize food resources.

Efficient foraging by *M. rubra* is mediated, in part, by the trail pheromone 3-ethyl-2,5-dimethylpyrazine ('EDP'), which is secreted by worker ants from their poison gland (Evershed et al., 1982). Groups of *M. rubra* workers readily follow trails of EDP in

laboratory bioassays (Evershed et al., 1982; Hoefele et al., 2021), confirming its functional role as a trail pheromone component. Evidence that *M. rubra* colonies in field bioassays did not readily recognize and follow experimental trails of EDP (Hoefele et al., pers. comm.) implies that the *M. rubra* trail pheromone could be more complex than previously thought, containing components other than just EDP. These components may be produced in, and secreted from, the poison gland, the Dufour's gland, or both, as reported in many other myrmicine ants (Cerdá et al., 2014). Extracts of poison and Dufour's glands contain many compounds (Cammaerts et al., 1978, 1981; Morgan & Wadhams, 1972), but their potential role as trail pheromone components have not yet been rigorously tested.

Depositing trail pheromone that guides nestmates to fleeting or rapidly diminishing food sources is an effective foraging tactic by ants. Pheromone trails leading to sustained food sources are more permanent and well maintained, and may further be defined by visual landmarks and chemical 'signposts' that ants refer to during foraging (Czaczkes et al., 2015; Steck, 2012). Such established trails are subject to eavesdropping by (heterospecific) ant community members that seek to exploit resources (Adams et al., 2020). In a recent study (Chalissery et al., 2019), we specifically tested the hypotheses that ant community members (*M. rubra*; *C. modoc*; black garden ants, *Lasius niger*) sense, and follow, each other's trail pheromones, and that they fail to recognize trail pheromones of allopatric ants (Argentine ants, *Linepithema humilis*; desert harvester ants, *Novomessor albisetosus*; pavement ants, *Tetramorium caespitum*). As *M. rubra* displaces many ant species (Naumann & Higgins, 2015), we expected that *M. rubra* would exploit pheromone trails of community members and outcompete them for use of food resources. We prepared the trail pheromones currently known for these six species of ants in a synthetic blend ('6SPB') and tested this blend for olfactory perception and trail-following responses by ants. All sympatric workers (*M. rubra*, *C. modoc*, *La. niger*) were able to antennally sense con- and heterospecific trail pheromones within the 6SPB. In trail-following bioassays, singly-tested workers of *C. modoc* and *La. niger* followed the 6SPB trail for a similar distance (*C. modoc*), and a farther distance (*La. niger)*, than they followed their own trail pheromone. These data indicate that con- and heterospecific pheromones have no adverse effects on trail-following by *C. modoc* and have additive effects on trail-following by *La. niger*. Yet, *M. rubra* workers did not follow trails of their own trail pheromone

(EDP) nor trails of the 6SPB, thereby not revealing whether they follow pheromone trails of ant community members. While these results contrast reports that single workers of many ant species readily follow (synthetic) pheromone trails (Chalissery et al., 2019, 2022; Muscedere et al., 2012; Renyard et al., 2019), they could support the concept that physical activation or recruitment by nestmates (see above) is required to induce trail-following in *M. rubra* (Fedoseeva, 2015). Whether super-colony nestmates of *M. rubra* in North America (Naumann et al., 2017) exclusively engage in group foraging could experimentally be investigated by bioassaying groups of ants, or individual ants, for their trail-following responses.

Here, we tested the hypotheses that (1) the *M. rubra* trail pheromone comprises a blend of several components, (2) groups of *M. rubra* workers, but not individual ants, follow pheromone trails, (3) groups of *M. rubra* workers follow pheromone trails of ant community members farther than their own trail pheromone, and (4) trail pheromone expedites recruitments of foraging *M. rubra* ants in field settings.

3.3. **Methods and Materials**

3.3.1. **Experimental Insects**

Laboratory colonies of *M. rubra* were established in Spring – Fall of 2019 and 2020 from natural colonies located at Inter River Park (Latitude: 49°19’12” N, Longitude: 123°01’42” W; North Vancouver, BC, CA). Colonies were excavated and placed into plastic totes (41 × 29 × 24 cm; 58 × 43 × 31 cm) or glass aquaria (26 × 21 × 40.6 cm; 30.5 × 26 × 50.8 cm) that were housed in the Science Research Annex on the Burnaby campus (49°16′33″ N, 122°54′55″ W) of Simon Fraser University. The room temperature was set to 25 °C and the photoperiod to 12L:12D. The same substrate (sand, rocks, wood) in which field colonies resided was placed into totes or aquaria, serving as material within which to nest. The surface of this substrate served as the foraging area for the ants which were provisioned with food [apples; insect prey (German and American cockroaches, *Blattella germanica* and *Periplaneta americana*; mealworms, *Tenebrio molitor*; superworms, *Zophobas morio*)] three times per week. Water reservoir test tubes (10 – 40 mL) in the foraging area were replaced when they were empty or had any signs of fungal growth on the cotton plug. To sustain the moist microclimate that *M. rubra* prefers to inhabit, nest substrate was regularly sprayed with water.

3.3.2. Extractions of the ants' abdomen, poison gland, and Dufour's gland

To analyze chemical constituents present in abdominal glands, 1000 worker ants were cold-euthanized on dry ice, their abdomens severed and placed in a vial containing 1 mL of dichloromethane (DCM; EMD Millipore Corp., Billerica, MA, USA). After macerating the abdomens with a glass rod, syringe plunger, and a vortex mixer (Labnet International Inc., Edison, NJ, USA), the sample was filtered through a ~10-mg glass wool plug (5 × 3 mm diam.) in a glass pipette to obtain 'tissue-free' abdomen extract.

To determine chemical constituents specifically of the poison gland and the Dufour's gland, 10 glands of each type were processed as previously described (Chalissery et al., 2022), Briefly, using forceps, the stinger – with the poison gland and the Dufour's gland attached – was gently pulled and thereby dislodged so that it floated to the water surface. The two glands were identified by reference to figures from Hölldobler and Wilson (1990), and were separated using an insect pin, forceps, and dissection scissors. Poison and Dufour's glands were placed into separate vials filled with 500–1000 µL of DCM and kept on dry ice. Gland tissues were macerated with a glass stirring rod and a vortex mixer, extracts were then filtered (see above) and concentrated to 100 µL.

3.3.3. Gas chromatographic-electroantennographic detection (GC-EAD) analysis of abdominal extracts

Aliquots (1 ant equivalent (1 AE)) of abdominal extracts were analyzed by GC-EAD and GC-mass spectrometry (MS) (see below). Samples were injected in splitless mode (1-2 µL injection volume), using procedures and equipment modified from previous reports (Gries et al., 2002). Briefly, the GC-EAD system employed a Hewlett-Packard (HP) 5890 gas chromatograph (GC) fitted with a DB-5 GC column (30 m × 0.32 mm I.D., film thickness 0.25 µm; J & W Scientific, Folsom, CA, USA). Helium served as carrier gas (35 cm · s^{-1}) with the following temperature program: 100 °C for 1 min., then 20 °C · min^{-1} to 280 °C. The injector port and flame ionization detector (FID) were set to 260 °C. For GC-EAD recordings (N = 2), an antenna was carefully dislodged from the head of a worker ant and suspended between two glass capillary electrodes (1.0 mm outer diameter (0.58 mm inner diameter) × 100 mm; A-M Systems, Carlsborg, WA, USA)

adapted to accommodate the ant antenna (~1 mm in length) and filled with saline (Staddon & Everton, 1980). Antennal responses to compounds in the column effluvium – that was directly released into a stream of medical air (250 mL/min flow) continuously passing over the electrode-suspended antenna – were amplified with a custom-built amplifier and recorded on an HP 3392A integrator.

3.3.4. **GC-MS analysis of abdominal, poison gland, and Dufour's gland extracts**

The compounds in abdominal extracts that elicited a response from ant antennae (see Results) were considered candidate pheromone components (CPCs) and were analyzed by GC-MS in full-scan electron ionization mode, using an Agilent 7890B GC coupled to a 5977A MSD (Agilent Technologies Inc., Santa Clara, CA, USA) and fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm). The injector port was set to 250 °C, the MS source to 230 °C, and the MS Quad to 150 °C. Helium was used as a carrier gas at a flow rate of 35 cm s^{-1} with the following temperature program: 50 °C (held for 5 min), 10 °C min^{-1} to 280 °C (held for 10 min). To determine the structure of the CPCs, their mass spectra and retention indices (relative to aliphatic alkanes; Van Den Dool & Kratz, 1963) were compared with those of authentic standards that were synthesized in our laboratory. To quantify the amounts of CPCs in any of the extracts, their GC peak area was compared with that a synthetic standard prepared at 1 ng/µl. To trace the origin of the antennally-active compounds, poison gland extracts and Dufour's gland extract were analyzed separately.

3.3.5. **Sources of 3-ethyl-2,5-dimethylpyrazine and (*Z*,*E*)-α-farnesene, and synthesis of (*Z*,*E*)-α-homofarnesene**

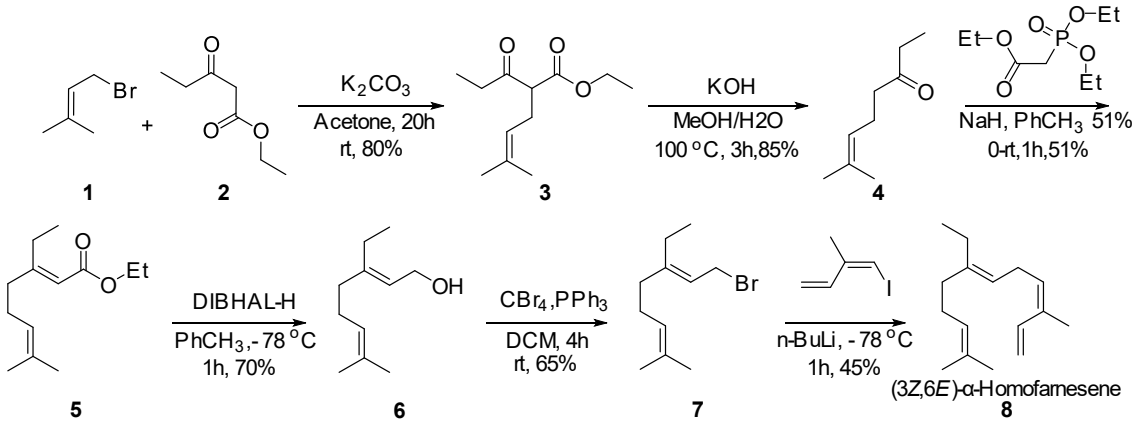
3-Ethyl-2,5-dimethylpyrazine (EDP) was purchased (Acros Organics, part of Thermo Fisher Scientific, New Jersey, USA) and bioassayed as an isomeric mixture (1:1) of 3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine (a non-natural isomer), drawing on reports that 3-ethyl-2,6-dimethylpyrazine has no adverse effects on trail-following responses of *M. rubra* (Hoefele et al., 2020). (*Z*,*E*)-α-Farnesene [(3*Z*,6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene] was synthesized following reported procedures (Ramaiah et al., 1995) and was available from a recent project (Gries et al.,

2022). (*Z*,*E*)-α-Homofarnesene [(3*Z*,6*E*)-7-ethyl-3,11-dimethyldodeca-1,3,6,10-tetraene] was synthesized as described below.

3.3.6. **General synthetic procedures**

All anhydrous reactions were performed under nitrogen atmosphere using flame-dried glassware. Normal-phase column chromatography was carried out with 230-400 mesh silica gel (Silicycle, 22SiliaFlash® P60). Trace solvents were concentrated and removed with a Büchi rotary evaporator. All reagents and starting materials were purchased from Sigma Aldrich, and were used without further purification. All solvents were purchased from Sigma Aldrich and EMD Millipore Corp., and were used without further purification, unless otherwise specified. Tetrahydrofuran (THF) was freshly distilled over Na metal/benzophenone. A temperature of -78 °C was obtained through an acetone-dry ice bath.

Specific syntheses



Scheme for the synthesis of (*Z*,*E*)-α-homofarnesene

Ethyl 5-methyl-2-propionylhex-4-enoate (3)

Prenyl bromide (716 mg, 4.71 mmol, 0.55 mL) and potassium carbonate (1.94 g, 14.1 mmol) were added to a 0.5 M solution of ethyl 3-oxopentanoate (700 mg, 4.71 mmol, 0.69 mL) in acetone After the reaction mixture was stirred 20 h at room temperature, it was quenched with 2 N hydrochloric acid. The organic layer was extracted with diethyl ether (2 × 25 mL), washed with brine, and dried over $MgSO_4$. Following solvent removal under reduced pressure, the residue was purified via silica gel flash column chromatography to obtain ethyl 5-methyl-2-propionylhex-4-enoate (**3**) as a light yellow oil

(797 mg; 80% yield). The spectral data of **3** matched those reported in the literature (Ondet et al., 2016).

7-Methyl-6-octen-3-one (4)

Potassium hydroxide (263 mg, 4,7 mmol) was added at room temperature to a stirred solution of ethyl 5-methyl-2-propionylhex-4-enoate (797 mg, 3.76 mmol) in ethanol/water (1:1). After stirring the mixture 3 h at reflux (100 °C), it was cooled to room temperature and extracted with diethyl ether (2 × 25 mL). Organic extracts were washed with water and brine and dried over $MgSO_4$. Following solvent removal under reduced pressure, the residue was purified via silica gel flash column chromatography to obtain 7-methyloct-6-en-3-one (**4**) as a yellow oil (447 mg; 85% yield). The spectral data of **4** matched those reported in the literature (Ondet et al., 2016).

Ethyl (E)-3-ethyl-7-methylocta-2,6-dienoate (5)

Ethyl 2-(diethoxyphosphoryl)acetate (0.86 mL, 3.8 mmol) was added to a suspension of NaH (84 mg, 3.5 mmol) in toluene (5 mL) at 0 °C. After 30 min, 7-methyl-6-octen-3-one (**4**; 447 mg, 3.19 mmol) was added dropwise. After stirring the reaction mixture 1 h under argon at room temperature, it was diluted with diethyl ether and water. The organic layer was separated and the aqueous phase extracted with diethyl ether (2 × 25 mL). The combined organic extracts were washed with brine and then dried over $MgSO_4$. Following solvent removal under reduced pressure, the residue was purified via silica gel flash column chromatography to obtain ethyl (*E*)-3-ethyl-7-methylocta-2,6-dienoate (**5**) as a yellow oil (335 mg; 51% yield). The spectral data of **5** matched those reported in the literature (Rawat & Gibbs, 2002).

Ethyl (E)-3-ethyl-7-methylocta-2,6-dien-1-ol (6)

Diisobutylaluminum hydride (1.5 M in toluene, 3.1 ml, 4.78 mmol) was added to a cooled (-78 °C) solution of ethyl (*E*)-3-ethyl-7-methylocta-2,6-dienoate (335 mg, 1.59 mmol) in toluene (5 mL). After keeping the reaction mixture 1 h at -78 °C it was quenched with a 40-mL aqueous solution of potassium sodium tartrate and the aqueous phase was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (35 mL) and dried over $MgSO_4$. Following solvent removal under reduced pressure, the residue was purified via silica gel flash column chromatography to obtain

ethyl (*E*)-3-ethyl-7-methylocta-2,6-dien-1-ol (**6**) as a yellow oil (187 mg; 70% yield). The spectral data of **6** matched those reported in the literature (Rawat & Gibbs, 2002).

(E)-1-bromo-3-ethyl-7-methylocta-2,6-diene (7)

CBr_4 (630 mg, 1.9 mmol) and Ph_3P (360 mg, 1.37 mmol) were added to a stirred solution of ethyl (*E*)-3-ethyl-7-methylocta-2,6-dien-1-ol (187 mg, 1.11 mmol) in 30 mL dichloromethane. After stirring the reaction mixture 4 h at room temperature, the solvent was removed and the resulting oil/solid taken up in hexanes. The suspension was cooled to and kept 30 min at -50 °C, following which it was filtered, concentrated, and resuspended. After repeating this procedure, the resulting oil was sufficiently pure for use in the next synthetic step. (*E*)-1-bromo-3-ethyl-7-methylocta-2,6-diene (**7**) was obtained as a brown oil (164 mg; 65% yield). The spectral data of **7** matched those reported in the literature (Rawat & Gibbs, 2002).

(Z,E)-α-Homofarnesene (8)

nBuLi (0.24 mL, 0.6 mmol, 2.5 M solution in hexanes) was added to a stirred solution of (*Z*)-1-iodo-2-methyl-1,3-butadiene (0.11 g, 0.55 mmol) in THF (2 mL) which was cooled to -78 °C. After stirring the resultant mixture 15 min at -78 °C, a solution of (*E*)-1-bromo-3-ethyl-7-methylocta-2,6-diene (115 mg, 0.5 mmol) in dry THF (2 mL) was added and the mixture was stirred 1 h at 23 °C. Then it was quenched with water, extracted with ether (2 × 25 mL), washed successively with brine, dried over $MgSO_4$, filtered, and concentrated. Flash chromatography on silica gel (with hexanes as eluent) afforded (*Z,E*)-α-homefarnesene as a colourless oil (49 mg; 45% yield).

3.3.7. **Circular trail-following experiment (laboratory setting)**

Following a previously reported experimental design (Chalissery et al., 2019, 2022; Renyard et al., 2019), experiments were run from July to August 2020. Briefly, all bioassays were performed below a metal scaffold (123 × 57 × 36 cm) enclosed in black fabric and lit from above with two lights (one plant light and one daylight fluorescent light, each 48″ 32 W F32T8; Phillips, Amsterdam, NL) to standardize visual cues during bioassays. Behavioral responses of ants were recorded using a phone video camera (Samsung S10e, Samsung Electronics, Suwon-si, South Korea) mounted above the arena. Using a micro-syringe, a test stimulus was applied as a continuous 25-cm long

trail 5 mm from the edge of a circular Whatman filter paper (90 mm diam.; Sigma-Aldrich), with the circumference marked with pencil in 1-cm intervals. This filter paper was then placed in a Pyrex glass Petri dish arena (150 mm diam.; Figure 3.1a).

In preparation for bioassays, a single ant or a group of five ants was isolated in a 1.5-mL micro-tube (Axygen™ MaxyClear Snaplock; Thermo Fisher Scientific, Waltham, MA, USA) and allowed 5 min to acclimate in the scaffold enclosure (see above). Five ants were deemed sufficient to generate a group effect while keeping the workload to analyze the behaviour of each ant within groups at a manageable level. To initiate a bioassay, a micro-tube was placed in the Petri dish arena so that its exit hole was ~1 cm away from the edge of the filter paper (Figure 3.1a) and the single ant, or group of ants, was given 10 min to leave the tube. Once the first ant had exited the micro-tube, the ants' behavior was video recorded 10 min. Video footage was analyzed using VLC Media Player (Version 2.2.6) to count the number of 1-cm intervals a trail-following single ant, or group of ants, had crossed during a bioassay, allowing us to determine the total distance ants had covered as a measure of orientation in response to the test stimulus (Chalissery et al., 2019; Morgan, 2009; Renyard et al., 2019). Ants that did not exit the micro-tube within 10 min were considered non-responders and were excluded from statistical analyses. For groups of ants, the mean distance covered by all responding ants (typically five or four out of five) was used to determine the trail-following response. For each replicate, a new filter paper was used, test stimuli were presented at 1 ant equivalent (1 AE) in 25 µl of pentane unless otherwise noted, and a new ant, or group of ants, was bioassayed.

Circular trail-following experiments (n = 10 each) tested the responses of single ants, and groups of ants, to one of four test stimuli (Table 1): (1) a 25-µl pentane control; (2) 3-ethyl-2,5-dimethylpyrazine ('EDP', 5 ng), the known trail pheromone component of *M. rubra;* (3) a 3-component trail pheromone blend ('3CPB') consisting of the EDP (5 ng), (*Z*,*E*)-α-farnesene (50 ng), and (*Z*,*E*)-α-homofarnesene (50 ng) (as quantified in 1 AE of poison and Dufour's gland extracts; and (4) a 6-species trail pheromone blend ('6SPB'), comprising trail pheromone components of *M. rubra* (EDP, 1 ng), *C. modoc* ((2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide, 7.5 ng; 2 AEs), *La. niger* (3,4-dihydro-8-hydroxy-3-7-trimethylisocoumarin, 0.5 ng), *T. caespitum* (2,5-dimethylpyrazine, 1 ng), *N. cockerelli* (4-methyl-3-heptanone, 10 ng), and *Li. humile* ((*Z*)-9-hexadecenal, 10 ng). The same number of replicates was run for each treatment on any bioassay day. Between

bioassays, preparative surfaces and the Petri dish arenas were cleaned with 70% ethanol and hexane, and the room was aired out for 5–10 min by opening an exterior door.

3.3.8. **Choice-of-trail experiment (field setting)**

To determine whether synthetic trail pheromone components of *M. rubra* affect nestmate recruitment in field settings, a choice-of-trail experiment (n = 10) was run in Inter River Park (Latitude: 49°19’12” N, Longitude: 123°01’42” W; North Vancouver, BC, CA) in September 2021. Not knowing the entrance to subterranean ant colonies and attempting to minimize the likelihood of recruiting the same foragers in sequential replicates, we (*i*) placed replicates in locations with significant ant activity, and (*ii*) spaced replicates at least 1.5 m apart along the forested edge of the field site (Figure 3.1b).

For each replicate, three strips of filter paper (25 × 2 cm each) were placed at 0º, 120º, and 240º (or at 60º, 180º, and 300º for every other replicate; Figure 3.1b) from a central point, taking care not to disturb the ants in the area. To prevent wind from moving strips, they were weighed down with several nails placed along the edges of strips. The distant end of each strip was baited with a small cylinder (~ 0.5 × 2 cm) of an ambrosia apple presented on a piece of paper (4.5 × 4.5 cm), to prevent ant access from below, and was protected from direct sun by a lid (16.2 cm diam.) of a Unitrap (Forestry Distributing, Boulder, CO, USA; Hoefele et al., 2021). After placing apple baits, the three test stimuli ((1) pentane (25 µL); (2) EDP (see above); (3) 3CPB (see above)) were randomly assigned to one of the three strips and applied with a micro syringe. To determine whether the EDP and the 3CPB expedite recruitment of nestmates to baits, photos of ants at food baits were taken at 10-min intervals for 50 min.

3.3.9. **Statistical analyses**

We analyzed all data with R 3.6.3 using packages “multcomp”, “plotrix”, “plyr”, “nlme”, “afex”, and “emmeans”. Data (counts of 1-cm increments followed along trails as a measure of “distance followed”) in the circular trail-following experiment were analyzed using a generalized linear model (GLM; quasi-Poisson distribution). The mean and standard error for distance followed by ants were analyzed by an analysis of variance (ANOVA), with Tukey’s honestly significant difference (HSD) tests for pairwise

comparisons of means run within ant cohort (single ants or 5-ant groups) across test stimuli (EDP, 3CPB, 6SPB, solvent control), and run within test stimuli across ant cohort. Data (number of foraging ants on food baits) in the choice-of-trail experiment (field setting) were analyzed using a linear mixed-effects (LME) model. The mean and standard error for number of foraging ants present at baits in response to test stimuli (EDP, 3CPB, solvent control) and time points (10-min intervals) were analyzed by a repeated measures ANOVA (with colony and location as random variables using the LME), with Tukey's HSD tests run within time points (across test stimuli).

3.4. Results

3.4.1. GC-EAD and GC-MS analyses of abdominal, poison gland, and Dufour's gland extracts

GC-EAD analyses of abdominal extracts revealed three compounds (**1**, **2** and **3** in Figure 3.2) that elicited responses from ant antennae. Their mass spectra suggested, and comparative GC-MS analyses with authentic standards confirmed, that they were EDP (**1**), (*Z*,*E*)-α-farnesene (**2**), and (*Z*,*E*)-α-homofarnesene (**3**). GC-MS analyses of poison gland and Dufour's gland extracts revealed that the EDP was present in the poison gland (at 5 ng per 1 AE), and (*Z*,*E*)-α-farnesene and (*Z*,*E*)-α-homofarnesene (each at 50 ng per 1 AE) were present in Dufour's gland (Figure 3.3).

3.4.2. Circular trail-following experiment (laboratory setting)

There was a significant effect of ant cohort and test stimuli on trail-following responses of worker ants (ANOVA; ant cohort, $F_1 = 18.99$, $P < 0.001$; test stimuli, $F_3 = 10.89$, $P < 0.001$; ant cohort × test stimuli, $F_3 = 0.7535$, $P = 0.52$; Figure 3.4).

Effect of test stimuli on trail-following responses of ant cohorts (singly-tested ants; 5-ant groups)

Based on Tukey's HSD tests, there was no significant difference in the distances that singly-tested ants followed trails of the solvent control (8.22 ± 2.15 cm), the EDP (18.40 ± 4.56 cm), the 3CPB (17.13 ± 6.41 cm), and the 6SPB (9.67 ± 4.49 cm) (EDP *vs*. 3CPB: $P = 1.00$; EDP *vs.* 6SPB: $P = 0.39$; EDP *vs.* solvent control: $P = 0.25$; 3CPB *vs.*

6SPB: $P = 0.55$; 3CPB *vs.* solvent control: $P = 0.37$; 6SPB *vs.* solvent control: $P = 0.99$; Figure 3.4).

Based on Tukey's HSD tests, there were significant differences in the distances that 5-ant groups followed trails of the solvent control (9.84 ± 1.49 cm), the EDP (34.32 ± 5.61 cm), the 3CPB (47.13 ± 4.31 cm), and the 6SPB (20.53 ± 4.89 cm). Distances followed by 5-ant groups differed between the solvent control and (*i*) the EDP ($P = 0.0029$) and (*ii*) the 3CPB ($P = 0.0001$). Distances followed also differed between the 3CPB and the 6SPB ($P = 0.01$). Other pairwise comparisons revealed no statistical differences (EDP *vs*. 3CPB: $P = 0.51$; EDP *vs.* 6SPB: $P = 0.25$; 6SPB *vs.* solvent control: $P = 0.23$; Figure 3.4).

Effect of ant cohort (singly-tested ants; 5-ant groups) on responses to test stimuli

Based on Tukey's HSD tests, five-ant groups followed trails of the EDP and the 3CPB farther (EDP: 34.32 ± 5.61 cm; 3CPB: 47.13 ± 4.31 cm) than singly-tested ants (EDP: 18.40 ± 4.56 cm; 3CPB: 17.13 ± 6.41 cm) (EDP: $P = 0.0300$; 3CPB: $P = 0.0010$; 6SPB: $P = 0.06$; solvent control $P = 0.71$; Figure 3.4).

3.4.3. **Choice-of-trail experiment (field setting)**

When *M. rubra* colonies in a field setting were offered choices between three paper strips treated with a trail of the EDP, the 3CPB, or the solvent control, with each trail leading to an apple bait (Figure 3.1b), there was a significant effect of test stimulus (pheromone *vs* control), and time point (10–50 min in 10-min intervals), on the number of ants recruited to apple baits (repeated measures ANOVA: test stimulus, $F_{(3,27)} = 41.12$, $P < 0.001$; time point, $F_{(4,109)} = 15.82$, $P < 0.001$; test stimulus × time point, $F_{(8,109)} = 3.62$, $P < 0.001$; Figure 3.5).

Ten minutes after experiment initiation, there were significant differences in the number of ants recruited to apple baits by the EDP (23.9 ± 4.40 recruits, the 3CPB (30.4 ± 5.87 recruits), and the solvent control (9.1 ± 2.58 recruits). Based on Tukey's HSD tests, numbers of ants at baits differed statistically between the 3CPB and the solvent control ($P = 0.0191$), but not between (*i*) the EDP and the solvent control ($P = 0.13$) and (*ii*) the EDP and the 3CPB ($P = 0.65$).

Similarly, 20 min after experiment initiation, there were significant differences in the number of ants recruited to apple baits by the EDP (28.3 ± 4.42 recruits), the 3CPB (39.7 ± 6.89 recruits) and the solvent control (14.7 ± 3.70 recruits). Based on Tukey's HSD tests, numbers of ants at baits differed statistically between the 3CPB and the solvent control ($P = 0.0056$), but not between (*i*) the EDP and the solvent control ($P = 0.17$) and (*ii*) the EDP and the 3CPB ($P = 0.28$).

At 30-, 40- and 50-min time intervals, there were no significant differences in the number of ants recruited to food baits by the EDP, the 3CPB, and the solvent control (30-min Tukey's HSD: EDP *vs*. 3CPB: $P = 0.87$; EDP *vs*. solvent control: $P = 0.35$; 3CPB *vs*. solvent control: $P = 0.16$; 40-min Tukey's HSD: EDP *vs*. 3CPB: $P = 0.82$; EDP *vs*. solvent control: $P = 0.85$; 3CPB *vs*. solvent control: $P = 0.50$; 50-min Tukey's HSD: EDP *vs*. 3CPB: $P = 0.99$; EDP *vs*. solvent control: $P = 0.92$; 3CPB *vs*. solvent control: $P = 0.87$).

3.5. **Discussion**

Our data support the conclusion that: (1) the trail pheromone of *M. rubra* consists of three components: 3-ethyl-2,5-dimethylpyrazine ('EDP'), (*Z*,*E*)-α-farnesene and (*Z*,*E*)-α-homofarnesene, with the two farnesenes modestly contributing to the pheromonal activity of this 3-component pheromone blend ('3CPB'); (2) groups of *M. rubra* worker ants, but not single ants, follow lines of trail pheromone; (3) worker ants follow 3CPB trails farther than they follow a trail pheromone blend ('6SPB') containing trail pheromone components of (*i*) *M. rubra,* (*ii*) community members of *M. rubra* (*La. niger* and *C. modoc*), and (*iii*) allopatric ant species; and (4) synthetic trails of the EDP and the 3CPB deployed in field settings expedite recruitment of *M. rubra* workers to food sources.

We have shown that the trail pheromone of *M. rubra* is more complex than previously reported. Findings in preliminary field bioassays that EDP trails did not elicit strong recruitment of nestmates to food baits (Hoefele et al., pers. comm.) made us hypothesize that the *M. rubra* trail pheromone contains components other than just the EDP. These additional components could be produced in, and secreted from, the poison gland, the Dufour's gland, or both (Cerdá et al., 2014). As these glands are difficult and time-consuming to collect, we opted first to prepare abdominal extracts of ants which

would contain all the constituents from these two glands. In GC-EAD analyses of abdominal gland extracts, not only EDP – the previously reported trail pheromone component of *M. rubra* (Evershed et al., 1982) – but also (*Z*,*E*)-α-farnesene and (*Z*,*E*)-α-homofarnesene elicited responses from worker ant antennae (Figure 3.2).

To determine the origin of the EDP and the two farnesenses in abdominal extracts, we prepared separate extracts of the poison gland and the Dufour's gland and analyzed them by GC-MS. These analyses demonstrated that the EDP originates from the poison gland, and the two farnesenes originate from the Dufour's gland (Figure 3.3), indicating that constituents from two different glands contribute to the composition of the *M. rubra* trail pheromone blend. While the secretion of EDP from the poison gland and its role as a trail pheromone component have been well documented (Evershed et al., 1982; Hoefele et al., 2021), no pheromonal activity had yet been assigned to the two farnesenes. The presence of α-farnesene and tentative identification of homofarnesene, as constituents of the *M. rubra* Dufour's gland, were previously reported (Morgan & Wadhams, 1972), but the specific isomer of α-farnesene was not yet known and unambiguous identification of homofarnesene as a gland constituent was still not complete. Here we report that (*Z*,*E*)-α-farnesene and (*Z*,*E*)-α-homofarnesene (*i*) originate from the Dufour's gland of *M. rubra* (Figure 3.3), (*ii*) elicit responses from worker ant antennae (Figure 3.2), and (*iii*) modestly contribute to the effectiveness of the trail pheromone blend (Figures 3.4, 3.5).

With evidence that groups of *M. rubra* workers readily follow EDP trails in laboratory settings (Evershed et al., 1982; Hoefele et al., 2021), but singly-tested workers did not (Chalissery et al., 2019), we hypothesized that that group recruitment of nestmates is essential for trail-following behavior in *M. rubra*. To rigorously test this hypothesis, we used a circular trail-following bioassay design (Figure 3.1a) and recorded the distance that 5-ant groups, or singly-tested ants, followed a test stimulus such as the EDP, the 3CPB, and the 6SPB. The data revealed that 5-ant groups, but not singly-tested ants, followed synthetic blends of trail pheromone components (Figure 3.4), supporting the concept that group recruitment or physical activation of nestmates by a forager is important for trail-following of *M. rubra* workers (Fedoseeva, 2015; Planqué et al., 2010).

Five-ant groups followed both EDP and 3CPB trails about 2-times farther than solvent control trails (Figure 3.4). However, only 3CPB trails prompted 5-ant groups to follow trails significantly farther than 6SPB trails, which were as ineffective as solvent control trails in eliciting trail-following responses (Figure 3.4). These data not only support the conclusion that (*Z*,*E*)-α-farnesene and (*Z*,*E*)-α-homofarnesene have a pheromonal function in the 3CPB and contribute to trail-following responses of ants, but they also indicate that the 6SPB contained constituents that interfered with trail-following responses of *M. rubra* workers. In addition to EDP, *M. rubra* antennae sense only three other components in the 6SPB (Chalissery et al., 2019): 3,4-dihydro-8-hydroxy-3-7-trimethylisocoumarin ('isocoumarin'), the trail pheromone of *La. niger*, (2S,4R,5S)-2,4-dimethyl-5-hexanolide ('hexanolide'), the trail pheromone of *C. modoc*, and 2,5-dimethylpyrazine, the trail pheromone of *T. caespitum*. That *M. rubra* antennae sensed the pyrazine pheromone of *T. caespitum* (allopatric species) is likely due to its structural similarity with the pyrazine pheromone component of *M. rubra*. It follows that it is the isocoumarin, hexanolide, or both that interfered with trail-following behavior of *M. rubra* workers in response to the 6SPB (Figure 3.4). While our data support the concept that ant community members (*M. rubra, La. niger, C. modoc*) sense, and respond to, each other's trail pheromones (Chalissery et al., 2019), the data did not support our prediction that the 6SPB would elicit trail-following responses by *M. rubra* workers as strong as, or stronger than, the EDP or the 3CPB. Our assumption that *M. rubra* workers would follow 6SPB trails (including pheromone components of sympatric *La. niger* and *C. modoc*) to learn about the location of rich food sources and then to exploit them may have been erroneous in that the foraging range of *M. rubra* colonies in North America is rather small (Higgins, pers. comm.), and eavesdropping on pheromone trails of ant community members may not accrue significant benefits. Alternatively, the presence of pheromone components in the 6SPB other than the EDP may simply have masked or 'diluted' the pheromonal message of the EDP.

Encouraged by our positive laboratory bioassay data (Figure 3.4), we also compared trail-following responses of *M. rubra* in a field setting, offering worker ants a choice between EDP, 3CPB, and solvent control trails, all leading to a food bait (Figure 3.1b). As trail pheromones serve to recruit nestmates to food sources (Czaczkes et al., 2015; Morgan, 2009), we scored the number of ants recruited to apple baits, rather than the distance ants followed trails (as in laboratory experiments), as the response criterion.

In this field experiment, both the 3CPB trail and the EDP trail recruited ants to apple baits significantly faster than the solvent control trail, but this differential recruitment effect quickly subsided over time (Figure 3.5). At both the 10-min and the 20-min timepoints, the 3CPB trail was superior to the EDP trail in that the numbers of ants at baits differed statistically between the 3CPB and the solvent control but not between the EDP and the solvent control (although the number of ants recruited by 3CPB and EDP trails did not differ statistically). Over time (30-min – 50-min timepoints), the number of ant recruits at control food baits increased, possibly because foragers avoided the crowded 3CPB and EDP trails, eventually found the food baits associated with the solvent control trails, and then deployed their own pheromone trails that recruited nestmates. That the recruitment effect of the 3CPB trail did not differ statistically from that of the EDP trail was likely due to the high ant activity in our field site. In sites of high ant activity, any food source – even without guiding trail pheromone – is quickly located by foraging ants that leave their nest from multiple exit points and then randomly encounter food sources like our experimental apple baits. Ultimately, this foraging strategy leads to concurrent exploitation of multiple food sources rather than a single established food source (Lehue et al., 2020; Lehue & Detrain, 2020). Our data interpretation is supported by findings in a field study with the pavement ant, *Tetramorium immigrans,* where the nestmate recruitment effect of synthetic trail pheromone was much more apparent in low- than in high-ant activity settings (Chalissery et al., 2022). Further testing in sites with low *M. rubra* infestation is needed to clearly reveal the differential recruitment effect afforded by EDP and 3CPB trails.

The chemical ecology of ant foraging behavior can be exploited for pest ant control. To this end, trail pheromone-based foraging behavior of ants could be used to guide pest ants to insecticide-laced food baits, where workers pick up the poisoned food, carry it to the nest, and share it – through trophallaxis – with nestmates, ultimately causing nest demise. Previous evidence that ant community members exploit each other's trail pheromone (Chalissery et al., 2019), provide added incentive to consider commercial development of trail pheromones for pest ant control. Trail pheromones of multiple species (such as the 6SPB) formulated in a single lure, with no antagonism and potential synergism between pheromone components, would make lure development commercially viable. In our study site, though, the population density of *M. rubra* was too high to demonstrate the profound effect of synthetic trail pheromone for expeditious

recruitment of nestmates. However, at early stages of new ant invasions when population densities are still low or immediately following population knockdowns through various control measures, deployment of synthetic trail pheromone to guide pest ants to lethal food baits may be very effective. To determine whether the deployment of multiple-species trail pheromone lures coupled with lethal food baits is a viable tactic for pest ant control, more studies of this concept are needed that target *M. rubra* and other major invasive ant pests such as the red imported fire ant, *Solenopsis invicta*, and the Argentine ant, *L. humile*.

In conclusion, the trail pheromone of *M. rubra* comprises not only 3-ethyl-2,5-dimethylpyrazine (EDP) (Evershed et al., 1982; Hoefele et al., 2021) but also (*Z*,*E*)-α-farnesene, and (*Z*,*E*)-α-homofarnesene, with EDP originating from the poison gland and the two farnesenes from the Dufour's gland. In the context of trail pheromone-guided foraging, *M. rubra* workers respond in groups. Only groups of workers, but not single workers, followed lines of synthetic trail pheromones. These data add to a growing body of literature on group-foraging (Fedoseeva, 2015; Kolay et al., 2020; Orlova & Amsalem, 2019) and pheromone eavesdropping (Adams et al., 2020; Chalissery et al., 2019). More studies are needed to quantify the extent by which the two farnesenses enhance the pheromonal activity of the EDP, and by which heterospecific trail pheromones interfere, or not, with optimal trail-following behavior of *M. rubra* workers in response to their own trail pheromone.

3.6. **Acknowledgements**

We thank Adam Blake and Asim Renyard for advice on statistical analyses and graphical illustrations, Jonathan Petrov, Kenza Zobaidi, April Lin, Jasper Li, Kai Zhang, Kelvin Lau, Kristy Lok, Saif Nayani, Julienne Coloma, Srishti Kumar, Miele McGowan, Devon Rai, Jessica Chalissery, and Rosemary Vayalikunnel for help with ant care, and Sharon Oliver for some word processing.

3.7. **Funding**

This research was supported by a Graduate Fellowship from Simon Fraser University, the MPM Graduate Entrance Scholarship, the Thelma Finlayson Graduate Fellowship, the Dr. H.R. MacCarthy Graduate Bursary, and the Mildred Wells Scholarship from the

BC Council of Garden Clubs, to JMC. The research was further supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) - Industrial Research Chair to GG with BASF Canada Ltd. and Scotts Canada Inc. as the industrial sponsors.

3.8. **References**

Adams, R. M. M., Wells, R. L., Yanoviak, S. P., Frost, C. J., & Fox, E. G. P. (2020). Interspecific eavesdropping on ant chemical communication. *Frontiers in Ecology and Evolution*, *8*, 24. https://doi.org/10.3389/fevo.2020.00024

Attygalle, A. B., & Morgan, E. D. (1983). Identification of trail pheromone of the ant *Tetramorium caespitum* L. (Hymenoptera: Myrmicinae). *Naturwissenschaften*, *70*, 364–365. https://doi.org/10.1007/BF00990315

Beckers, R., Deneubourg, J. L., Goss, S., & Pasteels, J. M. (1990). Collective decision making through food recruitment. *Insectes Sociaux*, *37*(3), 258–267. https://doi.org/10.1007/BF02224053

Bestmann, H. J., Kern, F., Schäfer, D., & Witschel, M. C. (1992). 3,4-Dihydroisocoumarins, a new class of ant trail pheromones. *Angewandte Chemie International Edition in English*, *31*(6), 795–796. https://doi.org/10.1002/anie.199207951

Billen, J., & Morgan, E. D. (1998). Pheromone communication in social insects: Sources and secretions. In *Pheromone Communication in Social Insects* (pp. 3–33).

Cammaerts, M. C., Inwood, M. R., Morgan, E. D., Parry, K., & Tyler, R. C. (1978). Comparative study of the pheromones emitted by workers of the ants *Myrmica rubra* and *Myrmica scabrinodis*. *Journal of Insect Physiology*, *24*(3), 207–214. https://doi.org/10.1016/0022-1910(78)90036-7

Cammaerts, M., Evershed, R., & Morgan, E. (1981). Comparative study of the Dufour gland secretions of workers of four species of *Myrmica* ants. *Journal of Insect Physiology*, *27*(1), 59–65.

Cammaerts-Tricot, M. C. (1978). Recruitment to food in *Myrmica rubra* L. *Biology of Behaviour*, *4*, 159–172.

Cerdá, X., van Oudenhove, L., Bernstein, C., & Boulay, R. R. (2014). A list of and some comments about the trail pheromones of ants. *Natural Product Communications*, *9*(8), 1934578X1400900. https://doi.org/10.1177/1934578X1400900813

Chalissery, J. M., Gries, R., Alamsetti, S. K., Ardiel, M. J., & Gries, G. (2021). Identification of the trail pheromone of the pavement ant *Tetramorium immigrans* (Hymenoptera: Formicidae). *Journal of Chemical Ecology*. https://doi.org/10.1007/s10886-021-01317-3

Chalissery, J. M., Renyard, A., Gries, R., Hoefele, D., Alamsetti, S. K., & Gries, G. (2019). Ants sense, and follow, trail pheromones of ant community members. *Insects*, *10*(11), 383. https://doi.org/10.3390/insects10110383

Czaczkes, T. J., Grüter, C., & Ratnieks, F. L. W. (2015). Trail pheromones: An integrative view of their role in social insect colony organization. *Annual Review of Entomology*, *60*(1), 581–599. https://doi.org/10.1146/annurev-ento-010814-020627

Evershed, R. P., Morgan, E. D., & Cammaerts, M. C. (1982). 3-Ethyl-2,5-dimethylpyrazine, the trail pheromone from the venom gland of eight species of *Myrmica* ants. *Insect Biochemistry*, *12*(4), 383–391. https://doi.org/10.1016/0020-1790(82)90035-X

Fedoseeva, E. B. (2015). A technological approach to the description of group foraging in the ant *Myrmica rubra*. *Entomological Review*, *95*(8), 984–999. https://doi.org/10.1134/S0013873815080060

Garnas, J., Groden, E., & Drummond, F. A. (2014). Mechanisms of competitive displacement of native ant fauna by invading *Myrmica rubra* (Hymenoptera: Formicidae) populations. *Environmental Entomology*, *43*(6), 1496–1506. https://doi.org/10.1603/EN14079

Gries, R., Herk, W., Alamsetti, S. K., Catton, H., Meers, S., Otani, J., & Gries, G. (2022). (*Z* ,*E*)-α-Farnesene—Sex pheromone component of female click beetle *Selatosomus aeripennis destructor* with intra- and inter-sexual communication function. *Entomologia Experimentalis et Applicata*, eea.13142. https://doi.org/10.1111/eea.13142

Gries, R., Khaskin, G., Gries, G., Bennett, R. G., King, G. G. S., Morewood, P., Slessor, K. N., & Morewood, W. D. (2002). (Z,Z)-4,7-Tridecadien-(*S*)-2-yl acetate: Sex pheromone of Douglas-fir cone gall midge, *Contarinia oregonensis*. *Journal of Chemical Ecology*, *28*, 2283–2297. https://doi.org/10.1023/A:1021005517389

Hicks, B. J. (2012). How does *Myrmica rubra* (Hymenoptera: Formicidae) disperse in its invasive range? Record of male-only swarming flights from Newfoundland. *Myrmecological News*, *16*, 31–34.

Hicks, B. J., & Marshall, H. D. (2018). Population structure of *Myrmica rubra* (Hymeno-ptera: Formicidae) in part of its invasive range revealed by nuclear DNA markers and aggression analysis. *Myrmecological News*, *28*, 35–43. https://doi.org/10.25849/myrmecol.news_028:035

Hoefele, D., Chalissery, J. M., Gries, R., & Gries, G. (2021). Effects of trail pheromone purity, dose, and type of placement on recruiting European fire ants, *Myrmica rubra*, to food baits. *Journal of the Entomological Society of British Columbia*, *117*, 31–41.

Hoefele, D., Chalissery, J. M., Renyard, A., & Gries, G. (2021). Experimentally guided development of a food bait for European fire ants. *Entomologia Experimentalis et Applicata*, *169*, 780–791. https://doi.org/10.1111/eea.13053

Hölldobler, B., Oldham, N. J., Morgan, E. D., & König, W. A. (1995). Recruitment pheromones in the ants *Aphaenogaster albisetosus* and *A. cockerelli* (Hymenoptera: Formicidae). *Journal of Insect Physiology*, *41*(9), 739–744. https://doi.org/10.1016/0022-1910(95)00041-R

Hölldobler, B., & Wilson, E. O. (1990). *The ants*. Belknap Press of Harvard University Press.

Keeling, C. I., Plettner, E., & Slessor, K. N. (2004). Hymenopteran semiochemicals. In S. Schulz (Ed.), *The chemistry of pheromones and other semiochemicals I* (Vol. 239, pp. 133–177). Springer Berlin Heidelberg. https://doi.org/10.1007/b95452

Kolay, S., Boulay, R., & d'Ettorre, P. (2020). Regulation of ant foraging: A review of the role of information use and personality. *Frontiers in Psychology*, *11*, 734. https://doi.org/10.3389/fpsyg.2020.00734

Lehue, M., & Detrain, C. (2020). Foraging through multiple nest holes: An impediment to collective decision-making in ants. *PLOS ONE*, *15*(7), e0234526. https://doi.org/10.1371/journal.pone.0234526

Lehue, M., Detrain, C., & Collignon, B. (2020). Nest entrances, spatial fidelity, and foraging patterns in the red ant *Myrmica rubra*: A field and theoretical study. *Insects*, *11*(5), 317. https://doi.org/10.3390/insects11050317

Morgan, E. D. (2009). Trail pheromones of ants. *Physiological Entomology*, *34*(1), 1–17. https://doi.org/10.1111/j.1365-3032.2008.00658.x

Morgan, E. D., & Wadhams, L. J. (1972). Chemical constituents of Dufour's gland in the ant, *Myrmica rubra*. *Journal of Insect Physiology*, 1125–1135.

Muscedere, M. L., Johnson, N., Gillis, B. C., Kamhi, J. F., & Traniello, J. F. A. (2012). Serotonin modulates worker responsiveness to trail pheromone in the ant *Pheidole dentata*. *Journal of Comparative Physiology A*, *198*(3), 219–227. https://doi.org/10.1007/s00359-011-0701-2

Naumann, K., & Higgins, R. J. (2015). The European fire ant (Hymenoptera: Formicidae) as an invasive species: impact on local ant species and other epigaeic arthropods. *The Canadian Entomologist*, *147*(5), 592–601. https://doi.org/10.4039/tce.2014.69

Naumann, K., Sá, M. M. D., Lewis, E., & Noronha, R. (2017). Supercolonies of the invasive ant, *Myrmica rubra* (Hymenoptera: Formicidae) in British Columbia, Canada. *Journal of the Entomological Society of British Columbia*, *114*, 56–64.

Ondet, P., Lempenauer, L., Duñach, E., & Lemière, G. (2016). Bismuth(III) triflate-catalysed tandem cyclisations towards complex polycyclic ethers. *Organic Chemistry Frontiers*, *3*(8), 999–1003. https://doi.org/10.1039/C6QO00212A

Orlova, M., & Amsalem, E. (2019). Context matters: Plasticity in response to pheromones regulating reproduction and collective behavior in social Hymenoptera. *Current Opinion in Insect Science*, *35*, 69–76. https://doi.org/10.1016/j.cois.2019.07.004

Planqué, R., van den Berg, J. B., & Franks, N. R. (2010). Recruitment strategies and colony size in ants. *PLoS ONE*, *5*(8), e11664. https://doi.org/10.1371/journal.pone.0011664

Ramaiah, P., Pegram, J. J., & Millar, J. G. (1995). A short synthesis of (3*Z*,6*E*)-α-farnesene. *The Journal of Organic Chemistry*, *60*(19), 6211–6213. https://doi.org/10.1021/jo00124a046

Rawat, D. S., & Gibbs, R. A. (2002). Synthesis of 7-Substituted Farnesyl Diphosphate Analogues. *Organic Letters*, *4*(18), 3027–3030. https://doi.org/10.1021/ol026176i

Renyard, A., Alamsetti, S. K., Gries, R., Munoz, A., & Gries, G. (2019). Identification of the trail pheromone of the carpenter ant *Camponotus modoc*. *Journal of Chemical Ecology*, *45*(11–12), 901–913. https://doi.org/10.1007/s10886-019-01114-z

Staddon, B. W., & Everton, I. J. (1980). Haemolymph of the milkweed bug *Oncopeltus fasciatus* (Heteroptera; lygaeidae): Inorganic constituents and amino acids. *Comparative Biochemistry and Physiology Part A: Physiology*, *65*(3), 371–374. https://doi.org/10.1016/0300-9629(80)90046-8

Steck, K. (2012). Just follow your nose: Homing by olfactory cues in ants. *Current Opinion in Neurobiology*, *22*(2), 231–235. https://doi.org/10.1016/j.conb.2011.10.011

Van Den Dool, H., & Kratz, P. Dec. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography A*, *11*, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X

Van Vorhis Key, S. E., & Baker, T. C. (1982). Trail-following responses of the Argentine ant, *Iridomyrmex humilis* (Mayr), to a synthetic trail pheromone component and analogs. *Journal of Chemical Ecology*, *8*(1), 3–14. https://doi.org/10.1007/BF00984000

Vander Meer, R. K., & Alonso, L. E. (1998). Pheromone directed behaviour in ants. In R. K. Vander Meer, M. D. Breed, K. E. Espelie, & M. L. Winston (Eds.), *Pheromone communication in social insects* (pp. 159–192).

3.9. **Tables**

Table 3.1 Names of test stimuli, components and amounts present in test stimuli, and ant species producing stimulus components

Test stimulus	Stimulus components (amount tested; Ant Equivalents (AEs))	Species	Reference
EDP[1]	3-Ethyl-2,5-dimethylpyrazine (5 ng; 1 AE)	*M. rubra*	Evershed et al., 1982
3CPB[2]	3-Ethyl-2,5-dimethylpyrazine (5 ng; 1 AE) (3*Z*,6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene ['(*Z*,*E*)-α-Farnesene'; 50 ng; 1 AE]; (3*Z*,6*E*)-7-ethyl-3,11-dimethyldodeca-1,3,6,10-tetraene ['(*Z*,*E*)-α-Homofarnesene'; 50 ng; 1 AE])	*M. rubra*	Evershed et al., 1982
6SPB[3]	3-Ethyl-2,5-dimethylpyrazine (5 ng; 1 AE) (2*S*,4*R*,5*S*)-2,4-Dimethyl-5-hexanolide (7.5 ng; 2 AEs) ('Hexanolide') 3,4-Dihydro-8-hydroxy-3-7-trimethylisocoumarin (0.5 ng; 1 AE) ('Isocoumarin') 4-Methyl-3-heptanone (10 ng; 1 AE) 2,5-Dimethylpyrazine (1 ng; 1 AE) (*Z*)-9-Hexadecenal (10 ng; 1AE)	*M. rubra* *C. modoc* *La. niger* *N. cockerelli* *T. caespitum* *Li. humile*	Evershed et al., 1982 Renyard et al., 2019 Bestmann et al., 1992 Hölldobler et al., 1995 Attygalle & Morgan, 1983 Van Vorhis Key & Baker, 1982

[1]EDP = 3-Ethyl-2,5-dimethylpyrazine; [2]3CPB = 3-component pheromone blend; [3]6SPB = 6-species pheromone blend

3.10. **Figures**

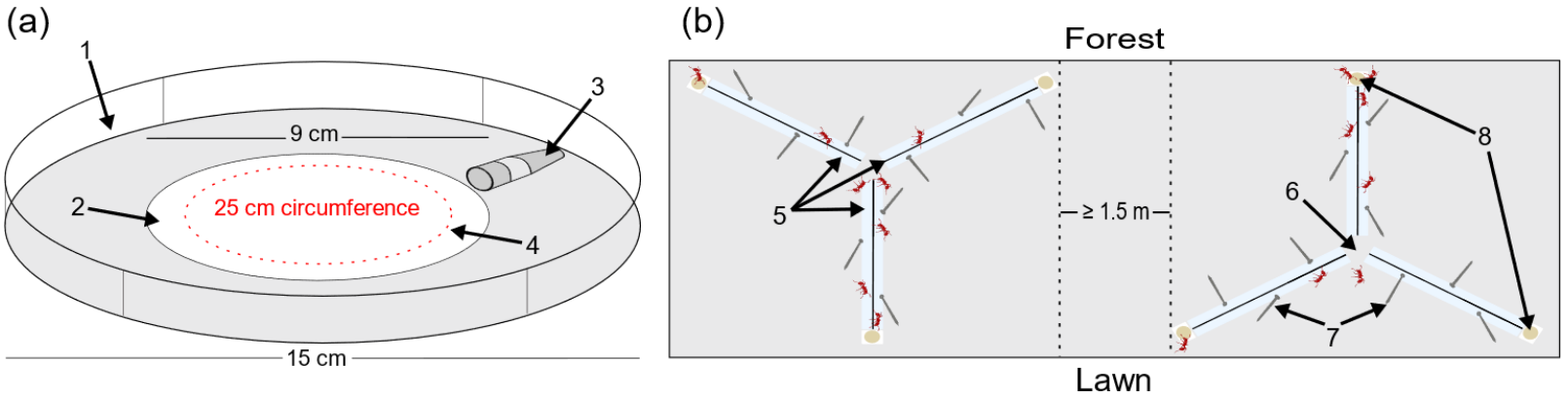


Figure 3.1 Graphic illustrations (not to scale) of the experimental design used for (a) laboratory-based circular trail-following experiments, and (b) a field-based choice-of-trail experiment. Design components for the circular trail-following experiments consisted of a Pyrex petri dish arena (1) fitted with a circular filter paper (2) marked in 1-cm intervals along the edge, a holding tube (3) from which a single ant, or a group of 5 ants, enters the arena, and a 25-cm long stimulus trail (4; dotted line). Design components of the choice-of-trail field experiment consisted of three paper strips (5; 25 × 2 cm each) treated with a test stimulus and placed at 0°, 120°, and 240° (or at 60º, 180º, and 300º for every other replicate) from a central point (6), nails (7) securing the strips to the ground, and apple baits (8) at the distant end of strips

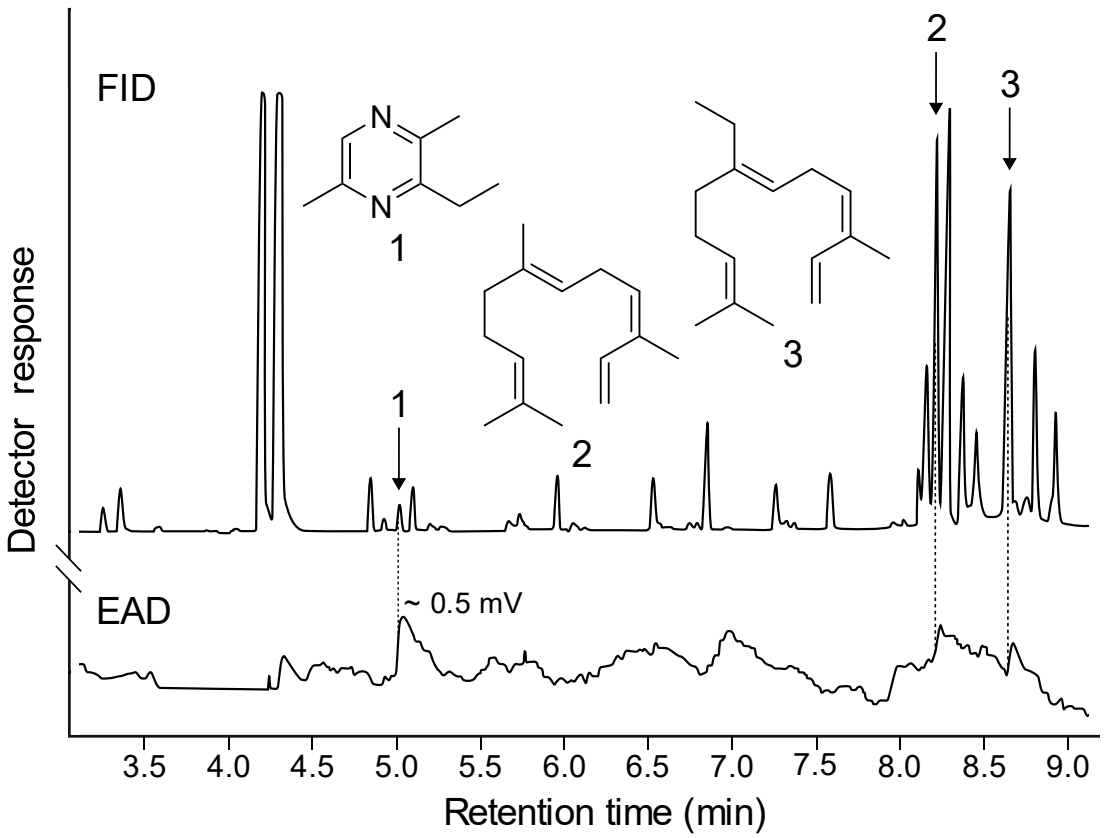


Figure 3.2 Representative recordings (n = 2) of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: antenna of a *Myrmica rubra* worker ant) to candidate trail pheromone components in abdomen extracts of *M. rubra* worker ants. FID peaks marked by arrows and numbers were identified as 3-ethyl-2,5-dimethylpyrazine (1), (*Z*,*E*)-α-farnesene (2), and (*Z*,*E*)-α-homofarnesene (3)

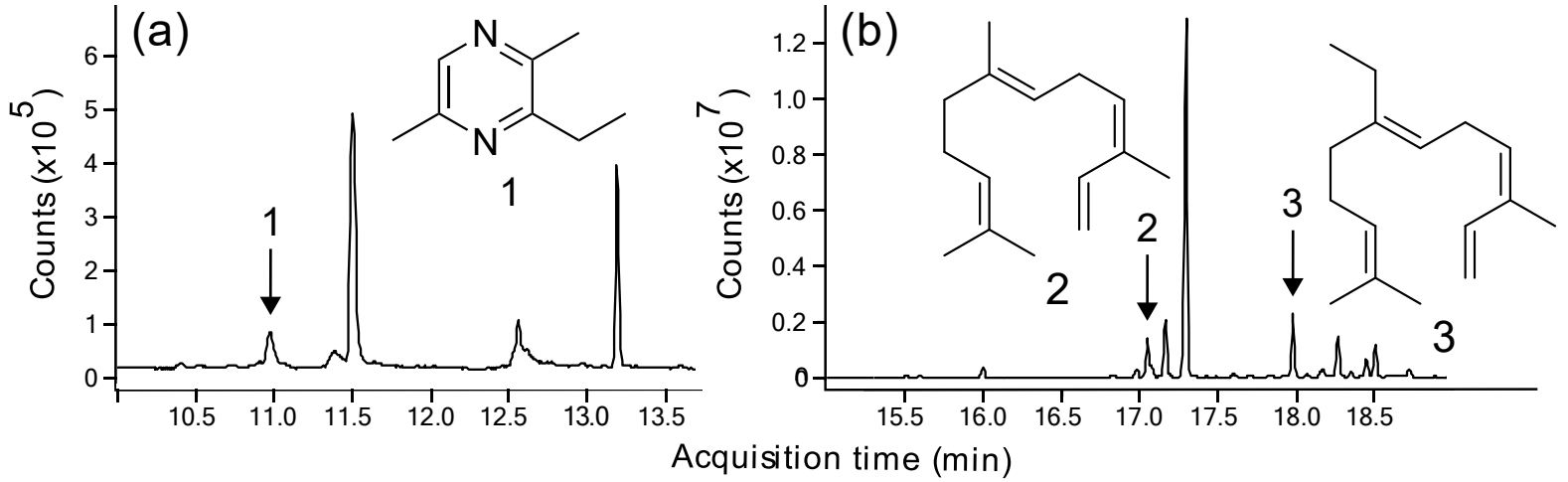


Figure 3.3 Total ion chromatograms of extracts of *Myrmica rubra* abdominal glands, revealing the presence of (a) 3-ethyl-2,5-dimethylpyrazine (1) in the poison gland, and (b) both (*Z*,*E*)-α-farnesene (2) and (*Z*,*E*)-α-homofarnesene (3) in the Dufour's gland

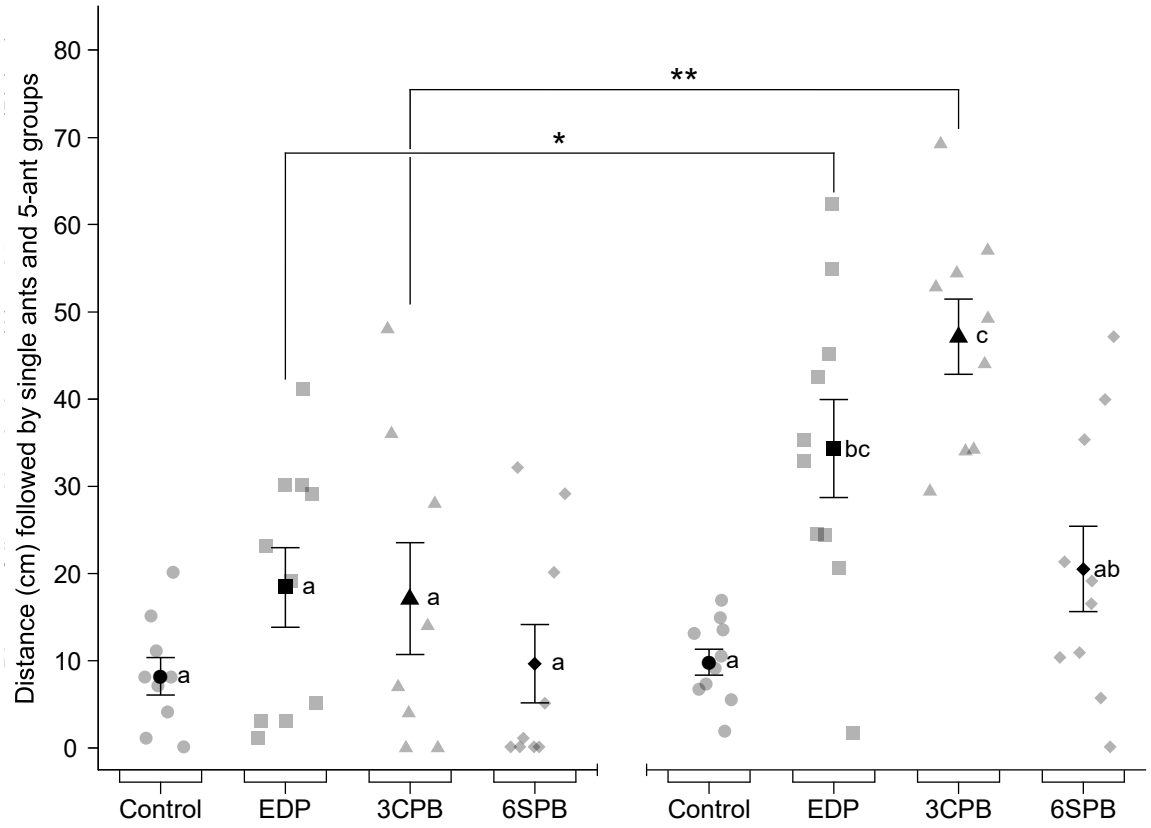


Figure 3.4 Distances worker ants of *Myrmica rubra* followed trails in circular trail-following experiments (design in Figure 3.1a). Grey and black symbols show the distance that singly-tested ants (left subpanel), and 5-ant groups (right subpanel), on average (mean ± standard error) followed trails. Test stimuli consisted of (1) a 25-µl pentane control ('Control'); (2) 3-ethyl-2,5-dimethylpyrazine ('EDP', 5 ng); (3) a 3-component trail pheromone blend ('3CPB') including EDP (5 ng), (*Z*,*E*)-α-farnesene (50 ng) and (*Z*,*E*)-α-homofarnesene (50 ng); and (4) a 6-species trail pheromone blend ('6SPB'), comprising trail pheromone components of *M. rubra* (EDP; 5 ng), *Camponotus modoc* ((2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide, 7.5 ng), *Lasius niger* (3,4-dihydro-8-hydroxy-3-7-trimethylisocoumarin, 0.5 ng), *Tetramorium caespitum* (2,5-dimethylpyrazine, 1 ng), *Novomessor cockerelli* (4-methyl-3-heptanone, 10 ng), and *Linepithema humile* ((*Z*)-9-hexadecenal, 10 ng). Within singly-tested ants (left subpanel), and within 5-ant groups (right subpanel), means associated with the same letter are not statistically different (Tukey's HSD tests, P < 0.01). Overarching, asterisk-marked lines indicate statistically significant differences in the responses of singly-tested ants and 5-ant groups to the same test stimulus (Tukey's HSD tests, * = P < 0.05, **= P < 0.01)

Note: (1) in each replicate of 5-ant group bioassays, the total distance travelled by all ants was divided by the number of travelling ants (those that left the holding tube); (2) stimuli were tested at 1 ant equivalent (AE; amount found within one worker ant) except for the hexanolide produced by *C. modoc* which was tested at 2 ant equivalents; (3) individual compounds, and blends of compounds, were presented in 25 µL of pentane applied to filter paper.

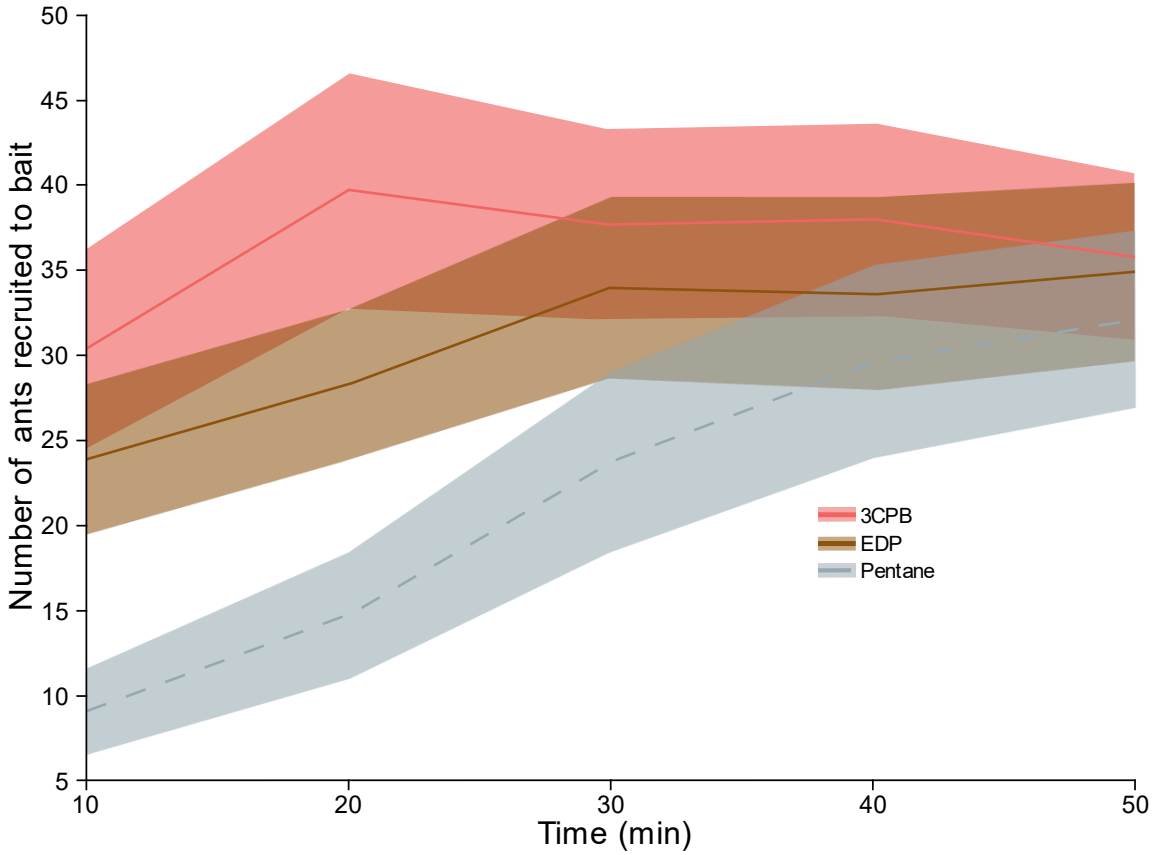


Figure 3.5 Mean numbers (solid and dotted lines) and standard errors (shaded areas around lines) of *Myrmica rubra* worker ants recruited by test stimuli to apple baits in a field experiment (design in Figure 3.1b). Test stimuli were applied to three paper strips radiating out from a central point. Test stimuli consisted of (1) a 25-µl pentane control ('Control'); (2) 3-ethyl-2,5-dimethylpyrazine ('EDP', 5 ng); and (3) a 3-component trail pheromone blend ('3CPB') including EDP (5 ng), (*Z*,*E*)-α-farnesene (50 ng) and (*Z*,*E*)-α-homofarnesene (50 ng). Data analyses revealed a significant effect of test stimulus (Control; EDP; 3CPB) and time point (10-min intervals between 10–50 min) on the number of ants recruited to apple baits (repeated measures ANOVA: treatment, $P < 0.001$; time point, $P < 0.001$; test stimulus × time point, $P < 0.001$)