Studying the Foraging and Communication Ecology of European Fire Ants

by

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Abstract

The European fire ant (EFA), or ruby ant, *Myrmica rubra* L., is an invasive pest in Greater Vancouver, British Columbia, Canada. EFAs are a nuisance to humans, swarming and stinging aggressively when nests are disturbed. They also cause ecological damage by altering invertebrate communities. The overarching goal of this thesis was to create a control method for EFAs. My specific research objectives were to: (1) develop an effective and affordable food bait; (2) determine trail following of EFAs in response to synthetic trail pheromone; and (3) determine trail following of ants in response to synthetic trail pheromone blends of multiple ant species. Food baits comprising diverse macronutrients such as carbohydrates (apples), proteins and lipids (dead insects) elicited the strongest foraging responses by EFAs. Re-hydrated freeze-dried baits proved as appealing as fresh baits and superior to rehydrated heat-dried baits. Isomerically pure and impure synthetic trail pheromone (3-ethyl-2,5-dimethylpyrazine) prompted similar recruitment responses of ants. The presence of pheromone, irrespective of dose tested, enhanced the recruitment of ants to food baits, with the dose of 200 ant equivalents eliciting the strongest recruitment responses. Trail pheromone applied in a line leading toward the food bait, but not in a circle surrounding it, was effective in recruiting ants, suggesting that 3-ethyl-2,5-dimethylpyrazine has a guiding but not an attractive function to EFAs. The presence of con- and hetero-specific pheromones had additive or indifferent effects on trail-following responses of garden ants, *Lasius niger,* and carpenter ants, *Camponotus modoc*, respectively. These data provide key information for the development of a highly functional insecticidal food bait for EFAs and other nuisance ant species.

Keywords: European Fire Ant; Myrmica; Formica; Integrated Pest Management; Chemical Ecology; Invasive Species

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Introduction

Invasive ants are a common and well documented phenomenon and problem worldwide (Holway et al. 2003). When ants invade new ecological niches, they often take over entire ecosystems and displace native ant species (Holway et al. 2003). Most invasive ants originate from the tropics, but the European Fire Ant (EFA), *Myrmica rubra*, is a temperate zone species that has become established in many regions across temperate North America (Holway et al. 2003). It has been postulated that climate zones with fewer native ant species, such as temperate climate zones, are extremely vulnerable to invasion by ants (Hölldobler & Wilson 1990). In their invasive range, EFAs are causing dramatic ecological changes (Naumann & Higgins 2015), while being a huge nuisance pest to humans (Higgins 2011). In this Introductory Chapter, I will examine the problem of invasive EFAs in North America and review available control methods.

Identification, appearance and taxonomy

Multiple ant species are commonly referred to as “fire ants” including the red imported fire ant, *Solenopsis invicta*, little fire ant, *Wasmannia auropunctata*, and the crazy ant, *Nylanderia fulva*. All three species are invasive in parts of their range (Callcott & Collins 1996; MacGown & Layton 2010; Wetterer & Porter 2003). Therefore, it is important to determine the species that requires attention. Instructions for “fire ant” control without specifying the species, as often seen (D.H., pers. obs.), are not very helpful.

EFAs are typically < 5 mm in length. They can be reddish-brown, red, or orange. They have a two-segmented petiole, two backwards-facing spines on their propodium, and heavy sculpting patterns on their frontal lobes which do not cover the base of their antennae (Arevalo & Groden 2007). Unlike other *Myrmica* species in British Columbia, EFAs have a notably flat space on the clypeus between the torulus (base of antenna)

and anterior edge of the clypeus. All other species have a sharp or rounded edge between the two (Higgins, pers. comm.).

EFA life cycle

EFAs are both polygynous and polydomous. Multiple reproductive queens reside in each nest, and a single nest may occupy several separate micro-locations (Elmes 1973). Nests typically consist of a few hundred workers and several queens (Fokuhl et al. 2007). An average of 15 queens per nest, with as many 670 queens per nest, was reported in England (Elmes, 1974; Groden & Drummond 2005). Queen numbers vary drastically over seasons (Brian et al. 1981). This phenomenon has implications for pest management because every single queen must be killed to effectively eliminate a nest. Polygynous ant species have historically been very successful invaders (Passera 1994).

EFAs build their nest in moist soil under, or partially within, woody debris or leaf litter, under concrete paving blocks, or in raised garden beds (Higgins 2015). Nests can be found in diverse habitats, including lawns, gardens, wetlands, deciduous forests, and along the edges of coniferous forests (Groden & Drummond 2005). Nests lack the conspicuous “ant hill” that is so characteristic of formicine ant nests (Arevalo & Groden 2007).

EFAs enter diapause during the winter. They are among the first temperate ants to resume foraging in the spring (Groden et al., unpublished data cited in Garnas 2004) and will keep foraging even during inclement weather such as heavy rain (D.H., pers. obs.). This tenacious foraging behavior makes EFAs strong intra- and interspecific competitors (Garnas 2004, 2014).

In their native range, EFAs produce both rapid brood and diapause brood. Rapid brood gives rise to worker ants in the same season, whereas diapause brood overwinters before it gives rise to workers, queens and males in the following season (Elmes 1982; Elmes & Wardlaw 1983; Kipyatkov & Lopatina 1997). The overlap of these two brood types leads to very high brood density in late June (Elmes 1982; Elmes et al. 1999). In their invasive range, EFAs produce diapause brood but it is not clear whether they also produce rapid brood (Arevalo & Groden 2007). EFAs forage more actively and are more of a nuisance when they have lots of brood in their nest. Conversely, when

they have little brood, their foraging activity is low and their nests are more difficult to detect (D.H., pers. obs.).

In their native range, EFAs typically engage in nuptial flights around August or September (Brian et al. 1981). New winged (alate) queens and alate males emerge from nests and take flight. They mate in flight, with the males eventually dying. Mated queens then either start new nests or enter existing nests, where they ablate their wings. At any time during the foraging season, EFAs also create new nests through “budding”, whereby a group of queens and workers with brood leave the old nest and start a new nest nearby (Elmes 1980). These “budding” ants are wingless and cannot move very far.

In their invasive range, EFAs do not seem to engage in nuptial flights. Alate males are typically observed from early July through September but alate queens are rarely seen (Groden & Drummond 2005). As queens become more numerous in late summer, many of them may be new queens that have already ablated their wings inside the nests. Alate queens emerging in late summer have never been observed taking flight (D.H., pers. obs.) and alate males were observed flying in only a single incident (Hicks 2012). Combined, all these accounts suggest that EFAs in their invaded North American range do not, or only rarely, engage in nuptial flights and produce new nests primarily or exclusively by budding. This interpretation is consistent with field observations of EFA infestations that are being completely halted at a stream or concrete sidewalk, despite suitable habitat on the other side (Groden & Drummond 2005). The same rationale would also explain the very high local EFA densities across their invaded range. A similar phenomenon occurs in the invasive Argentine ant, *Linepithema humile*, where nuptial flights are extremely rare and male-biased (Markin 1970).

The nest-budding tactic of EFAs in their invaded range results in multi-colonial infestations, with nest mates tolerant towards close neighbors but aggressive towards distant ones. Specifically, within a 10-m nest radius, nest mates tolerate non-nest mates but beyond that radius meet them with significant aggression (Garnas 2004). More recent research provides evidence for massive “supercolonies” (Naumann 2017), suggesting that there may be no aggression to ants from very distant nests.

The combination of polygyny, polydomy, nest budding and tolerance towards adjacent nests are all factors making the EFA a serious invasive pest (Garnas 2004,

2014) that readily colonizes entire parks and gardens, and completely alters the very fabric of the ecosystems it has invaded.

Diet and foraging

EFAs are omnivorous scavengers and predators. They prey mostly on invertebrates, and obtain some carbohydrates from plant exudates and hemipterans (Reznikova & Panteleeva 2001). EFAs frequently tend hemipterans such as aphids from which they collect honeydew but don't seem to be strongly associated with a specific species (Helms & Vinson 2008). They also collect seed elaiosomes and disperse seeds (Fokuhl et al. 2007).

As excellent foragers, EFAs can locate resources faster than other ant species (Holway 1999; Garnas 2014). How they outcompete other ants is not quite clear. Like other ants, EFAs deploy a trail pheromone to coordinate foraging activities (Evershed et al. 1982; Vander Meer et al. 1998). When a foraging EFA has found a profitable food source, she returns to the nest exuding from her poison gland and depositing the trail pheromone 3-ethyl-2,5-dimethylpyrazine. Other ants then use this trail to find their way to the food source, reinforcing the trail themselves. In general, ants deposit a stronger trail if they have found a high-quality food source (Vander Meer et al. 1998). Depositing and following a pheromone trail to a food source is common in ants but this tactic appears to be functioning particularly well in EFAs.

Range and habitat

The native range of EFAs includes most of Europe and central Asia (Fokuhl et al 2007). In this native range, EFAs are a nuisance but not a serious problem as they are in their invaded North American range.

EFAs were first detected in Massachusetts over 100 years ago (Groden & Drummond 2005; Wheeler, 1908). They are now present in British Columbia, Washington, Alberta, Saskatchewan, Ontario, Quebec, Nova Scotia, Newfoundland, New Brunswick, Prince Edward Island, Maine, Massachusetts, New Hampshire, New York, Rhode Island, and Vermont (Naumann & Higgins, 2015; Wetterer & Radchenko 2010). EFA infestations on the West coast of North America are considered separate

and independent introductions (Higgins 2013). EFAs are thought to have entered North America on imported plant material and indeed have been intercepted more recently on plants shipped from Europe (Groden & Drummond 2005). EFA infestations are more frequent in areas disturbed by humans but the precise effect of human disturbance, initial EFA nest size, and plant community composition on the presence and growth of EFA populations are all still not known (Groden & Drummond 2005). As EFAs do not fly in their invaded range, their long-distance spread is entirely reliant on humans. Since 2005, reports of EFAs as pests in North America have become more numerous (Groden & Drummond 2005), possibly due to several factors including local population adaptations and expansions, global warming, or simply greater public awareness.

The large habitat range of EFAs across Europe and central Asia implies pre-adaption to their invaded range (Elmes et al. 1999), with limited need for further physiological adaptations to new climates. In their native range, EFA populations are constrained by food supply and interspecific competition (Uchmanski & Petal 1982). Overall, it appears that EFAs have great potential to expand in their invaded range.

The impact of invasive EFAs

Economic and social issues

Invasive EFAs tend to occur in extremely high population densities, and appear far more aggressive than their counterparts in Europe. These characteristics enable them to wipe out native ant species (Naumann & Higgins 2015) and to render gardens, lawns and recreational areas unusable. Multiple nests may occupy an area as small as one square meter (D.H., pers. obs.). Nesting in such extreme density may allow them to improve their cold tolerance, escape natural enemies, or exploit more resources (Naumann & Higgins 2015).

EFA nests are difficult to spot and thus often accidentally stepped on. When disturbed, EFAs swarm and sting aggressively (D.H., pers. obs.). Their sting is painful with a pain level comparable to that of a milder wasp sting or to that of stinging nettles (Rob Higgins, pers. comm.). Multiple stings hurt drastically more than individual stings. After a day or two, sites of stings become swollen and itchy, significantly worse than mosquito bites (D.H., pers. obs.). There has been at least one person that required

hospitalization for anaphylactic shock treatment because of fire ant stings (Saltman, 2016).

As long-distance spread of EFAs is linked to inadvertent transportation by humans, EFAs tend to occupy areas inhabited by humans (Groden & Drummond 2005). People owning private property and businesses are often unwilling to disclose EFA infestations for fear that their property value will decline (Rob Higgins, pers. comm.).

Ecological effects

EFAs in their invaded range have a particularly severe impact on native ant species. A study showed that pitfall traps placed in invaded areas captured >99.9% EFAs (Naumann & Higgins 2015). Moreover, EFA captures in EFA-infested sites were 10- to 1300-fold higher than captures of any other ants in sites void of EFAs. According to comparative studies on multiple ant species, EFAs seem to be more efficient foragers and better nest defenders than most other ant species, thus enabling them to outcompete native ants (Garnas 2004, 2014). By virtue of numbers, EFAs can overwhelm even those ants that are typically strong defenders of their resources (Holway 1999). In the Lower Mainland of British Columbia, the biodiversity of all arthropods was significantly lower in EFA-infested sites (Naumann & Higgins 2015). However, the diversity and richness of “ant-limited” hemipterans may increase in EFA-infested habitats (Garnas 2004), because hemipterans benefit from EFA tending behaviour.

EFAs may also have a significant effect on vertebrates, particularly ground-nesting birds. In Maine (USA), EFAs displace herring gulls, *Larus argentatus*, decrease their nesting efficiency, and occasionally kill their chicks (DeFisher & Bonter 2013).

Conversely, EFAs in their native range are considered ecologically important and are even protected due to their relationship with Maculinea butterflies that parasitize their nests (Elmes et al. 1998).

Management of EFAs

The few available tactics for control of EFAs are inefficient and/or costly, and some are still being developed.

Prevention

Preventing the influx of invasive pests is easier than eliminating them once established. Property owners should carefully inspect purchased plants and soil for pest presence before putting them on their property. Open communication with neighbors can be extremely helpful to prevent inadvertent introduction of many pest species. If there is a risk of EFA invasion, some precautions can be taken to prevent it.

Peppermint, spearmint and neem extracts are repellent to EFAs (Bernard 2014) and can be applied to pots and raised beds to prevent EFAs from moving in from adjacent properties. Spearmint and peppermint oils are considered minimum risk pesticides and are exempted from registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) of the United States. However, these repellents have been tested only in small scale, short term bioassays, and more research is necessary to determine their effectiveness on a large scale. Neem oil is currently not registered for pest control in Canada. Cedar mulch has been tested as an EFA repellent but was found not effective (Bernard 2014).

If there is an imminent risk of EFA invasion, microhabitats that are potential EFA nesting sites (moist soil, woody debris or leaf litter, concrete paving blocks, raised garden beds (Higgins 2015)) should be removed or rendered unsuitable. As EFAs favor moist soil, watering should be kept to a minimum. Also, as EFAs prefer long grass, lawns should be cut short (Higgins, 2015).

Detection of EFAs

If EFAs are present on one's property, each nest must be located before control measures are implemented (Higgins 2017). Locating nests can be difficult because EFAs do not create visible nest mounds.

Nest locations can be approximated by laying out a grid of apple slices (1 slice per m^2) and by counting the number of forager ants on each slice 90 minutes later (Higgins 2017).

To further hone in on nest locations, areas can be purposefully disturbed, thereby prompting ants to swarm their nest entrance and mount a defense response (D.H., pers.

obs.). To implement a disturbance, wear shoes and clothing through which EFAs cannot enter or sting, select a 1-m^2 target area, and then slide your feet back and forth across the target area. If there is an EFA nest entrance in the target area, ants will swarm within a minute. EFAs that may have climbed onto your shoes or clothing do not cling tightly and can easily be shaken off. All nests should be marked before eradication attempts are made.

Control measures

Diatomaceous Earth

Diatomaceous earth can be applied as a barrier between EFA-infested and EFA-free sites (Higgins 2015) but it also kills non-target arthropods. Applications of diatomaceous earth help prevent the spread of EFAs but do not curtail current population densities. Diatomaceous earth is certified organic, free of chemical pesticides, and generally safe to humans and vertebrates. As rainfall renders diatomaceous earth ineffective, many repeat applications are necessary.

Digging and torching

Digging and torching EFA nests is a proven effective tactic to eliminate EFAs from entire sites (Higgins & Dessureault, pers. comm.). This tactic entails repeatedly turning over EFA-infested soil with a shovel while simultaneously burning the soil with a propane torch. While this tactic is effective and not reliant on pesticides, it is dangerous and labour-intensive. This tactic is appropriate for small EFA infestations on high-value properties.

Nematodes

Entomopathogenic nematodes have been tested several times without success for control of EFAs (Bernard 2014; Higgins 2015).

Trapping and Freezing

The trapping and freezing tactic exploits the tendency of EFAs to build their nests below paving or stepping stones. Soil surrounding a nest is excavated and a container lined with towels, landscaping cloth or paper is placed into the hole. The container lid should be flush with the soil surface and be covered with a paving stone. When EFAs have colonized a container, often within about a week (Higgins 2015), it can be removed, frozen, and then returned to eliminate another nest.

This tactic is suitable for longer-term management of serious EFA infestations. Because this tactic is labour-intensive and on its own not sufficiently effective, it should be combined with other control measures. A botanical garden had some success using trapping and freezing in combination with other tactics to reduce EFA populations to more manageable levels (Higgins, pers. comm.).

Baits

Poison food baits for ants are available from several companies. “Home remedy” baits consisting of 2% boric acid in sugar water also provide some control but do not eradicate ants. To obtain some level of EFA control, many poison food baits need to be deployed across infested areas. Boric acid is lethal to other invertebrates and toxic to vertebrates including humans (Boone et al. 2012).

Permethrins

Methodical destruction of nests by digging them up and treating them with permethrins is currently the best tactic for controlling EFAs on private properties. Excavated nests are placed into a bucket and sprayed with a 0.25%-permethrin solution. For successful elimination of EFAs from a private property, all nests must be located and treated within a short period of time. As permethrins are dangerous to fish and cats, precautions must be taken. This tactic is labour-intensive and may need to be repeated. Applying this tactic has resulted in the successful removal of EFAs from the meditation gardens at VanDusen botanical gardens (Higgins 2015)

Conclusion

The EFA is an invasive and widely distributed pest across North America causing serious ecological, economic, and social complications. The extreme density of their nests renders infested lawns, gardens, and parks unusable. The combination of polygyny, polydomy, nest-budding and reduced intraspecific aggression, all enable EFAs to completely take over ecosystems and to displace native ants (Garnas 2004, 2014; Naumann & Higgins 2015). EFAs fare well in human-disturbed locations, where they build nests in ideal microhabitats such as moist garden beds and under concrete slabs (Groden 2005). Only a few tactics are effective for EFA control, but they are labour-intensive and costly. Municipal governments should inform the public of the risk of spreading EFAs in soil and plant material. The likelihood of EFAs spreading across property lines and getting established on new properties can be minimized by applying repellants or insecticides at property borders and by removing potential nesting sites. Established nests can be removed by excavating and torching them, or by treating excavated nests with insecticidal permethrin (Higgins & Dessureault, pers. comm.). EFAs can also be encouraged to build nests in prepared containers that are subsequently removed and frozen (Higgins 2015). Poison food baits can be purchased or prepared using boric acid as the lethal agent in a sugar solution. Multiple control tactics applied concurrently are more likely to succeed at eradicating EFAs than any single tactic (Higgins, pers. comm.).

Overview of Research Chapters

The overarching goal for my MPM research was to study questions pertinent to the foraging and communication ecology of EFAs, with the ultimate objective to develop an EFA control tactic that couples an appealing (but poisonous) food bait with a trail recruitment pheromone leading ants toward it. The specific objectives of my research chapters 1-3 are described below.

Deployment of poisonous food baits for EFA control shows great promise. EFAs forage socially, share food by trophallaxis, and thus may spread the poison together with the food throughout their entire nest. Current food baits contain almost exclusively carbohydrates even though EFAs consume a wide range of food types (Reznikova & Panteleeva, 2001). **In research chapter 1**, I describe laboratory and field testing of food

baits with select macronutrients (carbohydrates, fat, protein) for their ability to attract and prompt feeding by EFAs. I further explore natural and affordable sources of macronutrients, and I test whether measures that help extend the shelf life of food baits (e.g., freeze drying followed by re-hydration prior to field deployment) have adverse effects on the attractiveness of food baits.

The efficacy of lethal food baits is enhanced by synthetic trail pheromone guiding foraging ants to these baits. The commercial source of the EFA trail pheromone, 3-ethyl-2,5-dimethylpyrazine, contains 2-ethyl-3,6-dimethylpyrazine as a non-pheromonal isomer which may, or may not, interfere with the ants' trail following behavior. Moreover, responses of ants to trail pheromone are typically dose-dependent (Evershed et al. 1982; Kohl et al. 2001, 2003; Morgan et al. 2006), with the highest pheromone concentration not always eliciting the strongest response (Morgan et al. 2006; Reynard et al. 2019). Finally, the type of trail pheromone placement (encircling a food bait or leading toward it) may alter the recruitment effect of nestmates dependent upon whether the trail pheromone has a guiding or attractive function. **In research chapter 2**, I test the effects of trail pheromone purity, dose, and type of placement on recruiting EFAs.

Pheromone trails leading to persistent and high-quality food sources are generally well maintained by foraging ants (Morgan 2009), and thus are readily exploited by eavesdropping (heterospecific) non-nestmates (Adams, 1990; Gobin, Peeters, Billen, & Morgan, 1998; Hölldobler & Wilson, 1990; Menzel, Pokorny, Blüthgen, & Schmitt, 2010; Vander Meer et al., 1998; Wilson, 1965) Aggressive encounters with non-nest mates on shared trails (Gobin et al., 1998; Hölldobler & Wilson, 1990; Menzel et al., 2010) are largely avoided by using dissimilar activity and foraging schedules. Mutual recognition of pheromone trails can be expected for co-evolved ant community member, unless an invading species has already become a well-established and integrated community member. **In research chapter 3**, I test the hypothesis - in collaboration with several of my labmates - that ant community members, including EFAs, in British Columbia's Lower Mainland sense, and behaviorally respond to, each other's trail pheromones but fail to recognize the trail pheromones of allopatric (non-community) ant species.

Chapter 2. Experimentally-guided development of a food bait for European fire ants*

*A near identical version of this chapter has been submitted for review to Entomologia Experimentalis et Applicata, with the following authors: Danielle Hoefele, Jaime Chalissery, Asim Renyard, Gerhard Gries; DH, JC, AR & GG conceived the study; DH & JC collected data; DH & JC analyzed data; DH & GG wrote the first draft; all authors reviewed and approved of the final draft for submission.

Chapter Abstract

Deployment of lethal food baits could become a control tactic for the invasive European fire ant (EFA), *Myrmica rubra* L. (Hymenoptera: Formicidae), because foraging ants carry the lethal food to their nest and share it with their nest mates, ultimately causing the demise of nests. Our objective was to develop a food bait with extended shelf life that is cost-effective to produce and elicits a strong foraging response from EFAs. To develop a bait composition with "ant appeal", we ran two separate field experiments testing pre-selected carbohydrate sources (oranges, apples, bananas) and protein/lipid sources [tuna, pollen, sunflower seeds, mealworms (*Tenebrio molitor*)]. While foraging EFAs responded equally well to the three types of carbohydrates, they preferred mealworms to all other protein/lipid sources. In a follow-up laboratory experiment, the combination of apples and mealworms elicited a stronger foraging response from EFAs than either apples or mealworms alone. To help reduce bait ingredient costs, we tested house crickets (*Acheta domestica L.*) as a less expensive mealworm alternative and found crickets and mealworms comparably appealing. Addressing the shelf life of baits, we tested freeze-dried and heat-dried apple/cricket combinations. Rehydrated freeze-dried baits proved as appealing as fresh baits and superior to rehydrated heat-dried baits, suggesting that freeze-drying may retain essential nutrients and/or aroma constituents. As freeze-drying is expensive, further research should investigate the preservation of moist food baits or the development of dry baits that are hydrated prior to deployment.

Introduction

Invasive ants cause serious ecological problems worldwide (Holway et al., 2002). Temperate climate zones are particularly vulnerable to invasion by ants (Hölldobler & Wilson, 1990) which often displace native ants and disrupt entire ecosystems (Holway et al., 2002). Most invasive ants originate from the tropics but the European fire ant (EFA), *Myrmica rubra* L., is a temperate zone species that is now established in many regions across temperate North America (Holway et al., 2002). EFAs are both polygynous and polydomous, with multiple reproductive queens residing in each nest, and a single nest possibly occupying several separate micro-locations (Elmes, 1973; Passera, 1994). In their invaded range, EFAs (*i*) do not engage in nuptial flights (Groden & Drummond, 2005) resulting in high nest densities, (*ii*) cause ecological havoc by altering invertebrate communities (Naumann & Higgins, 2015), and (*iii*) are nuisance pests to humans (Naumann & Higgins, 2015) swarming and stinging aggressively when disturbed (D.H., pers. obs.).

There are currently only a few methods available to control EFAs, and none is very effective. Excavating nests and spraying them with insecticide is work-intensive and must be repeated if not all queens are killed (Higgins, 2017). Moreover, topical insecticide treatments of nests reach only those ants that are above ground at the time of treatment. In contrast, the tactic of deploying lethal food baits to control EFAs shows promise because EFA workers carry (lethal) food back to their nest and share it, through trophallaxis, with their nest mates (Brian & Abbott, 1977), which could ultimately cause the demise of nests.

Like many other ant species (Raubenheimer & Simpson, 1999; Dussutour & Simpson, 2008), EFAs eat a diverse diet comprising carbohydrates, proteins and lipids. As omnivorous scavengers and predators, EFAs forage for proteins and lipids in form of live or dead invertebrates, and for carbohydrates in plant exudates and hemipteran excretions (Reznikova & Panteleeva, 2001; Naumann & Higgins, 2015). EFAs frequently obtain sugary honeydew from a variety of aphids (Helms & Vinson, 2008) and proteins and lipids from seed elaiosomes (Fokuhl et al., 2007).

EFAs are excellent foragers that quickly exploit resources (Holway, 1999; Groden et al., unpubl. data cited in Garnas, 2004). Like other ants, EFAs deploy a trail

pheromone to coordinate foraging activities (Vander Meer et al., 1998). After a foraging EFA has located a food source, she deposits a trail pheromone (3-ethyl-2,5-dimethylpyrazine) while returning to the nest (Evershed et al., 1982). Her nest mates then use this trail for guidance to the food source, possibly reinforcing the trail. Natural or synthetic trail pheromone likely enhances the effectiveness of lethal food baits (Greenberg & Klotz, 2000; Welzel & Choe, 2016; Hoefele et al., unpubl. data).

The development of lethal food baits for ants must take into account the ants' dietary needs, costs for bait ingredients and manufacturing, and the desired shelf life of baits. While long-term maintenance of laboratory ant colonies requires a complex diet with macro- and micro-nutrients (Straka & Feldhaar, 2007; Dussutour & Simpson, 2008) including vitamins and trace salts, lethal food baits should contain only those dietary constituents that optimize food uptake by ants and transport to the nest. The composition of currently marketed ant baits is typically proprietary and may or may not be based on rigorous testing of all ingredients for their appeal to foraging ants.

Shelf life is a critically important criterion for commercial ant baits which remain on shelves of retail stores for extended periods of time. Moist baits are optimal breeding grounds for microbes (Phillips et al., 1979) and require costly refrigeration to prevent deterioration. To extend shelf life, food products for human consumption are commonly dried. Drying is accomplished by a variety of techniques that differ in cost and effect on the food quality. Freeze-drying is expensive but largely retains the nutritional value and aroma of food products (Ratti, 2001). Heat-drying is more economic but may alter properties of food products (Ratti, 2001) and may remove food odorants that otherwise could attract ants to lethal food sources.

Aiming at the development of an effective and affordable food bait for EFAs, and ants in general, we assumed that all essential macronutrients (carbohydrates, proteins, lipids) need to be represented. We also took into account prior reports of food types that are readily accepted or selected by foraging ants, such as apples (R. Higgins, pers. comm.), meat products (Williams et al., 1980), pollen (Czechowski et al., 2008), myrmecochorous seeds (Fokuhl et al., 2007), seed elaiosomes (Fischer et al., 2008), and live prey or dead insects (Porter, 1989). We substituted myrmecochorous seeds with more affordable sunflower seeds, and dead insects with laboratory-reared

mealworms, *Tenebrio molitor L.,* and house crickets, *Acheta domestica L.*, which are mass-produced efficiently as animal feed (DeFoliart, 1989; Parajulee et al., 1993).

With the development of a commercially viable and effective ant bait in mind, our specific research objectives (O) were to: (O1) identify carbohydrate and protein sources (including dead mealworms) preferentially foraged upon by EFAs; (O2) test whether the preferred carbohydrate and protein source (see O1) in combination enhance foraging responses by EFAs; (O3) determine whether mealworms and (more affordable) crickets elicit comparable foraging responses by EFAs; and (O4, O5) investigate the effect of freeze-drying baits (O4), or heat-drying baits (O5), on foraging responses of EFAs.

Methods

Colony Collection

We followed a protocol previously described (Chalissery et al., 2019). Briefly, in three locations of the Lower Mainland of British Columbia, we excavated circa 30 cm^3 of soil surrounding the entrance of EFA nests and placed the nests in separate 19-L buckets. Wearing nitrile gloves for protection from stings, we sifted through the soil by hand and collected about 10 queens, 200-300 workers, and 50-100 larvae/pupae from each nest. We transferred these ants to “nest boxes” in our laboratory. Laboratory colonies were used in bioassays once they commenced to exhibited “near-natural” colony behavior (foraging for food, creating waste piles, living in the nest). Nests took 1-2 weeks to reach this point.

Rearing of experimental ants, mealworms and crickets

We kept ant colonies indoors in the Science Research Annex of Simon Fraser University (SFU) at a temperature of 25 °C and a photoperiod of 12L:12D. Each colony was housed in a nest-box (15 × 15 × 9 cm tall), partially filled with potting soil, and was given access to a plastic tote foraging arena (41 × 29 × 24 cm or 58 × 43 × 31 cm) fitted with a mesh-covered hole in the lid to allow air flow. Ants entered and exited the nest box through a 15-cm long Nalgene tubing (3 mm diam). The upper-most rim of each foraging arena was coated with a 1:1 mixture of paraffin oil and petroleum jelly to prevent ants from escaping. Twice per week, we provisioned each nest with a large

variety of proteinaceous and carbohydrate-type food sources to minimize the possibility that ants "learned" to forage for a specific food type. We provided water in a test tube fitted with a piece of cotton which was replaced whenever it became moldy or dry. Twice per week, we rehydrated nest-boxes by spraying water through the mesh window in the foraging arena.

We purchased mealworms from a private seller via Craigslist (https://vancouver.craigslist.org/), reared them in a plastic bin (61 × 39.5 × 21 cm high) with mesh-covered holes for ventilation, and provisioned them with potatoes, carrots, milk powder, oats, and wheat bran. When mealworms were sufficiently large and needed in experiments, they were removed from bins and frozen. House crickets were purchased (Noah's Pet Ark, Vancouver, BC, Canada) and 150 individuals kept in each of two glass aquaria (50 × 32 × 26 cm high) fitted with egg cartons and newspaper, and provisioned with dog food and a water source. When needed for experiments, adult crickets were frozen to prepare test stimuli.

Pre-screening food types for foraging responses of ants

To pre-select food sources for subsequent rigorous experimental testing, we offered EFAs a large variety of proteinaceous or carbohydrate-containing foods (canned tuna, cat food, dog food, tofu, canned beans, canned chicken, dehydrated shrimp, anchovy paste, cheese, dead mealworms, dead blow flies, sunflower seeds, pumpkin seeds, luncheon meat, canned oranges, apple slices, apple sauce, kiwis, grapes, candy, honey, sugar water, raisins), noting the food types heavily foraged on by ants. Based on these data, we then selected as carbohydrate sources apples, bananas and oranges, and as protein/lipid sources canned tuna, pollen, sunflower seeds, and mealworms.

General design of field experiments (Exps. 1, 2)

We field tested pre-selected food types (see above) between June and September 2016 at the Inter-River Park (North Vancouver, BC), where we established a line of 16 bait stations (Exp. 1), or 15 bait stations (Exp. 2), along the edge of a wooded area heavily infested with EFAs. Taking into account that EFAs forage only within about 2 m of their nest (Higgins, pers. comm.), we separated bait stations by 2 m, each station

at least 30 cm away from any EFA nest entrance. Statistically, each bait station was both a block and a replicate.

For each station, we randomly assigned one of three food baits or the unbaited control to one of four experimental days (Exp. 1), or one of four food baits or the unbaited control to one of five experimental days (Exp. 2). We presented each food bait in a 9-cm petri dish on top of damp cotton to account for moisture differentials between food types and to facilitate bait access by ants. We cut and ground (using a mortar and pestle) each food source into small pieces that could readily be carried by ants, presenting 2-gram equivalents for each food type and bait. To prevent baits from drying out or overheating, and to prevent birds from preying upon foraging ants, we placed the lid (16.2 cm diam.) of a Unitrap (Forestry Distributing Inc, Boulder, CO, USA) over each bait without affecting access of ants to baits (Fig. 2.1, A). Taking peak foraging activities of ants into account (as established in preliminary tests), we initiated experimental testing between 10:00 and 11:00 and terminated replicates 2 or 5 h later by quickly closing the Petri dishes with Petri dish lids and securing them with tape. Following cold-euthanization, the number of ants in each Petri dish were counted.

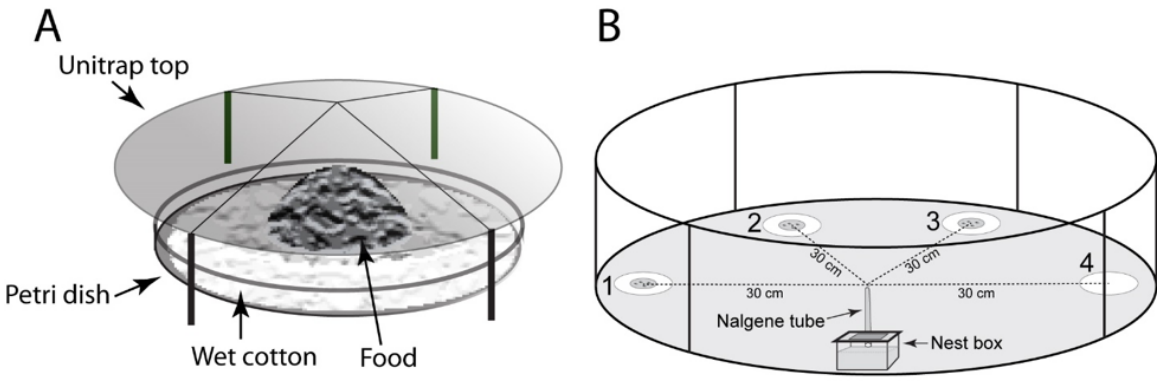

Figure 2.1. Graphical illustrations depicting (A) a trapping station deployed in field experiments 1 and 2, consisting of a Petri dish (9 cm diam) fitted with a moist cotton pad and baited with a food source; the moist cotton pad accounted for moisture differentials of food stimuli, and the Unitrap top provided protection from the sun and prevented birds from preying upon foraging ants; (B) a bioassay arena (122 cm diam × 40 cm high, used in laboratory experiments 3-8) fitted with (*i*) a nest box (15 × 15 × 9 cm tall) housing a European fire ant colony, and (*ii*) two or four test stimuli placed in the middle of a moist cotton pad.

General design of laboratory experiments (Exps. 3-8)

We ran laboratory experiments in six circular arenas (122 cm diam. × 40 cm high) housed in a dedicated bioassay room (22-23 ºC) in the Science Research Annex of SFU. We deprived colonies of food (but not water) 5-7 days prior to bioassays to enhance foraging propensity (without causing harm), testing each colony only once in each experiment. Statistically, each colony was both a block and a replicate. We prepared baits as described for field experiments but did not cover Petri dishes with a Unitrap lid.

To prepare for a bioassay, we temporarily closed the entrance tube to a nest box with a piece of cotton and then transferred the nest box from the foraging arena to a bioassay arena. We placed moist cotton pads (9 cm in diameter) 30 cm from the nest entrance in positions **1**, **2**, **3** or **4** for 4-choice bioassays, and in positions **1** or **4** for 2-choice bioassays (Fig. 2.1, B). In experiments with dried food baits, we placed the bait on a watch glass surrounded by a moist cotton ring. To initiate a bioassay, we opened the nest box entrance and allowed ants 6 h to forage, after which we counted ants present on food baits and terminated experimental replicates. Following bioassays, we collected ants still foraging by aspirator or hand, and returned them together with the nest box to their original foraging arena.

Specific Experiments

(O1) Experiment 1: Effect of carbohydrate sources on field-foraging responses of EFAs

We followed the general protocol for field experiments (see above), and in each of 16 replicates presented in random order on each of four days one Petri dish that was baited with (1) apples (Ambrosia, Canada), (2) bananas (Del Monte, Guatemala) and (3) oranges (Naval, Sunkist), or (4) that were kept unbaited (control) containing only moist cotton.

(O1) Experiment 2: Effect of protein/lipid sources on field-foraging responses of EFAs

We followed again the general protocol for field experiments (see above), and in each of 15 replicates presented in random order on each of five days one Petri dish that

was baited with (1) canned tuna (solid white tuna albacore in water, Clover Leaf Seafoods, Markham, ON, Canada) (2) pollen (obtained from bumble bees foraging on blueberry and wildflower), (3) raw hulled sunflower seeds (Dan-D Foods Ltd, Richmond, BC, Canada), and (4) mealworms, or (5) that were kept unbaited (control) containing only moist cotton.

(O2) Experiment 3: Interactive effect of a carbohydrate and protein source on lab-foraging responses of EFAs

With apples and mealworms preferentially sought by EFAs in (field) experiments 1 and 2 (see Results), we proceeded with laboratory experiments 3-8, following the general design described above. In experiment 3, we tested in circular arenas whether apples and mealworms in combinations enhance foraging responses of EFAs. In each of nine replicates, we presented four cotton pads that were baited with (1) apples, (2) mealworms, and (3) a blended mixture of apples and mealworms, or (4) that were kept unbaited (control) containing only moist cotton.

(O3) Experiment 4: Comparative effect of mealworms and crickets on lab-foraging responses of EFAs

Mealworms were the favoured protein source in experiment 2 (see Results) but unlike mealworms, crickets are already industrially reared and thus would be a cheaper insect protein source. For bioassays, insects were sliced into 2-3 pieces (depending on size) and ground with a mortar and pestle. In each of 14 replicates, we presented four cotton pads that were baited with (1) crickets, (2) mealworms, (3) a blended mixture of crickets and mealworms, or (4) that were kept unbaited (control) containing only moist cotton.

(O4) Experiments 5-7: Effect of freeze-drying baits on lab-foraging responses of EFAs

Sliced apples and crickets were weighed, kept 1 h in separate containers at -80 ºC, and then freeze-dried (VIRTIS Freezemobile 25L Freeze Dryer, SP Scientific, Warminster, PA, USA) for at least 4 days. Following freeze-drying, all samples were re-weighed to determine weight loss due to water loss, using these data to assign a water volume for rehydrating a cohort of freeze-dried samples (see below). All freeze-dried test stimuli were placed on a watch glass surrounded by a ring of moist cotton to prevent water uptake from the cotton. For experimental symmetry, a watch glass was present in

each Petri dish including the control. Prior to bioassays, Petri dishes with test stimuli were stored at 0 ºC for at least 1 h or until the bioassay commenced.

(O4) Experiment 5: Effect of freeze-drying crickets on lab-foraging responses of EFAs

In each of 16 replicates, we tested four treatments. We presented four watch glasses (surrounded by a ring of moist cotton) that were baited with (1) fresh crickets (2 g), (2) freeze-dried crickets (2 g), and (3) freeze-dried crickets (0.65 g) rehydrated with 1.35 mL (1.35 g) of water, or (4) that were kept unbaited (control).

(O4) Experiment 6: Effect of freeze-drying apples on lab-foraging responses of EFAs

In each of 21 replicates, we presented four watch glasses (surrounded by a ring of moist cotton) that were baited with (1) fresh apples (2 g), (2) freeze-dried apples (2 g), and (3) freeze-dried apples (0.68 g) rehydrated with 1.32 mL (1.32 g) of water, or (4) that were kept unbaited (control).

(O4) Experiment 7: Effect of freeze-drying apples and crickets on lab-foraging responses of EFAs

In each of 21 replicates, we presented four cotton pads with watch glasses (surrounded by a ring of moist cotton) that were baited with blended mixtures of (1) fresh apples (1 g) and fresh cricket (1 g), (2) freeze-dried apples (1 g) and freeze-dried cricket (1 g), and (3) freeze-dried apples (0.16 g) and freeze-dried crickets (0.25 g) rehydrated with 1.59 mL (1.59 g) of water, or (4) that were kept unbaited (control).

(O5) Experiment 8: Comparative effect of freeze-drying or heat-drying combined cricket and apple baits on lab-foraging responses of EFAs

We obtained heat-dried apple powder (North of 49 Naturals, Delta, BC, Canada) and heat-dried cricket powder (Coast Cricket Protein, New Westminster, BC, Canada). In each of 20 replicates, we presented two watch glasses (surrounded by a ring of moist cotton) that were baited with (1) cricket powder (0.5 g) and apple powder (0.2 g) rehydrated with 1.30 mL (1.3 g) of water, or that were baited with (2) freeze-dried apple (0.16 g) and freeze-dried crickets (0.25 g) rehydrated with 1.3 mL (1.3 g) of water.

Statistical analyses of data

Data were analyzed in JMP using a mixed effect analysis using block as a random effect and treatment as a fixed effect, with Tukey-Kramer pair-wise comparisons of means ($P < 0.05$), as indicated. Blocks in field experiments 1 and 2 were bait stations and in lab experiments 3-8 were ant colonies.

Results

(O1) Experiment 1: Effect of carbohydrate sources on field-foraging responses of EFAs

Carbohydrate baits had a significant effect on foraging responses of EFAs ($F_{3,15} = 6.34$, $P = 0.001$; Fig. 2.2, top) but there was no significant difference between treatment groups ($P > 0.05$). Petri dishes (Fig. 2.1, A) baited with apples, bananas or oranges attracted on average 3.5-, 3.1-, and 2.9-times more ants, respectively, than unbaited (control) petri dishes.

(O1) Experiment 2: Effect of protein/lipid sources on field-foraging responses of EFAs

Protein/lipid baits also had a significant effect on foraging responses of EFAs ($F_{4,14} = 16.08$, $P < 0.0001$; Fig. 2.2, bottom). Bait effectiveness differed in accordance with bait content. Petri dishes (Fig. 2.1, A) baited with mealworms attracted on average 1.7-, 1.9-, and 2.9-times more ants than the bait of seed, pollen and tuna, respectively. Mealworms were significantly more effective than seeds or tuna; statistically, the latter three baits were not different from one another, and tuna was not different from unbaited controls.

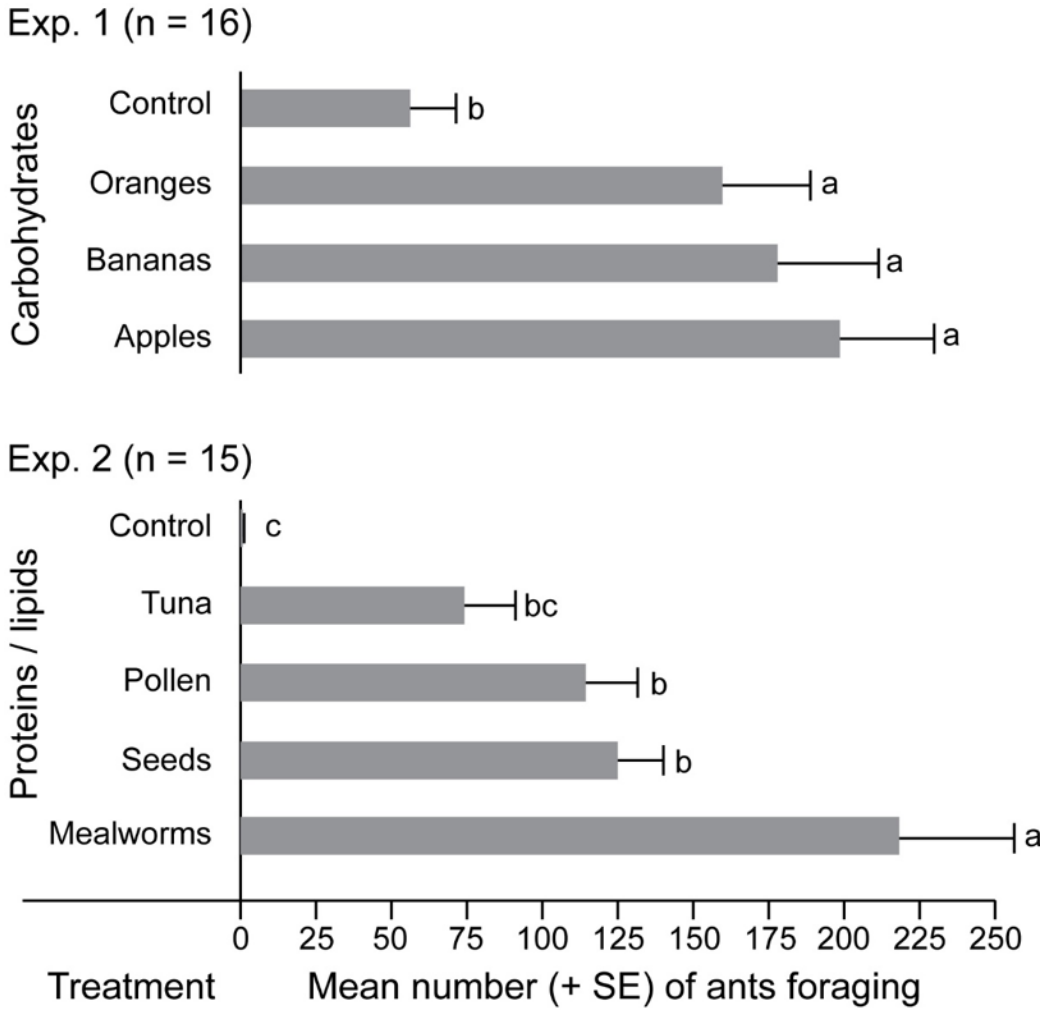

Figure 2.2. Mean numbers (+ SE) of European fire ants, *Myrmica rubra*, present in Petri dishes (Fig. 2.1, A) baited with carbohydrate or protein/lipid food types (2-g equivalents) in field experiments 1 and 2. In each experiment, bars with different letters indicate statistically different foraging responses by ants (Mixed effect analysis using block as a random effect and treatment as a fixed effect, with Tukey-Kramer pairwise comparisons of means; $P < 0.05$).

(O2) Experiment 3: Interactive effect of a carbohydrate and protein source on lab-foraging responses of EFAs

In laboratory arena bioassays (Fig. 2.1, B), bait composition had a significant effect on foraging responses of EFAs ($F_{3,8} = 13.61$, $P < 0.0001$; Fig. 2.3). Baits consisting of a blended mixture of apples and mealworms attracted on average 2.4- and 3.4-times more ants than mealworm-only and apple-only baits, respectively; statistically, mealworm-only and apple-only baits were not more effective than unbaited controls.

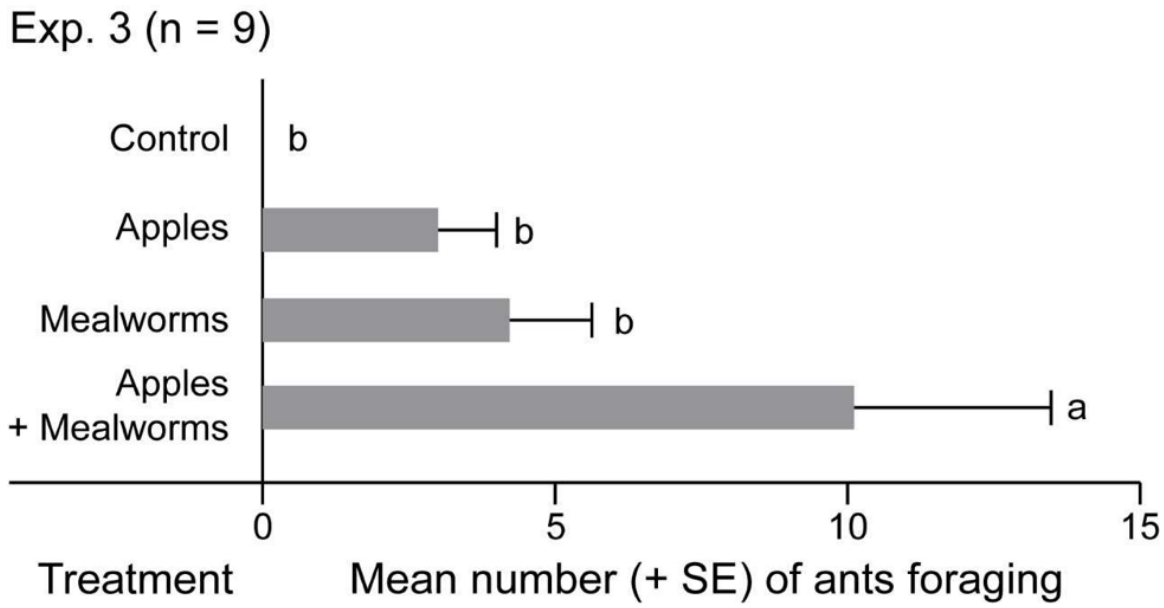

Figure 2.3. **Mean numbers (+ SE) of European fire ants, *Myrmica rubra*, present on test stimuli (2-g equivalents) in experiment 3. Bars with different letters indicate statistically different foraging responses by ants (Mixed effect analysis using nest as a random effect and treatment as a fixed effect, with Tukey-Kramer pairwise comparisons of means; $P < 0.05$).**

(O3) Experiment 4: Comparative effect of mealworms and crickets on lab-foraging responses of EFAs

In laboratory arena bioassays (Fig. 2.1, B), bait composition had a significant effect on foraging responses of EFAs ($F_{3,13} = 3.40$, $P = 0.027$; Fig. 2.4). Baits consisting of a blended mixture of mealworms and crickets attracted on average 1.3- and 1.6-times more ants than cricket-only and mealworm-only baits, respectively. Statistically, these effects were not significant. Similarly, cricket- and mealworm-only baits were not more effective than the unbaited control.

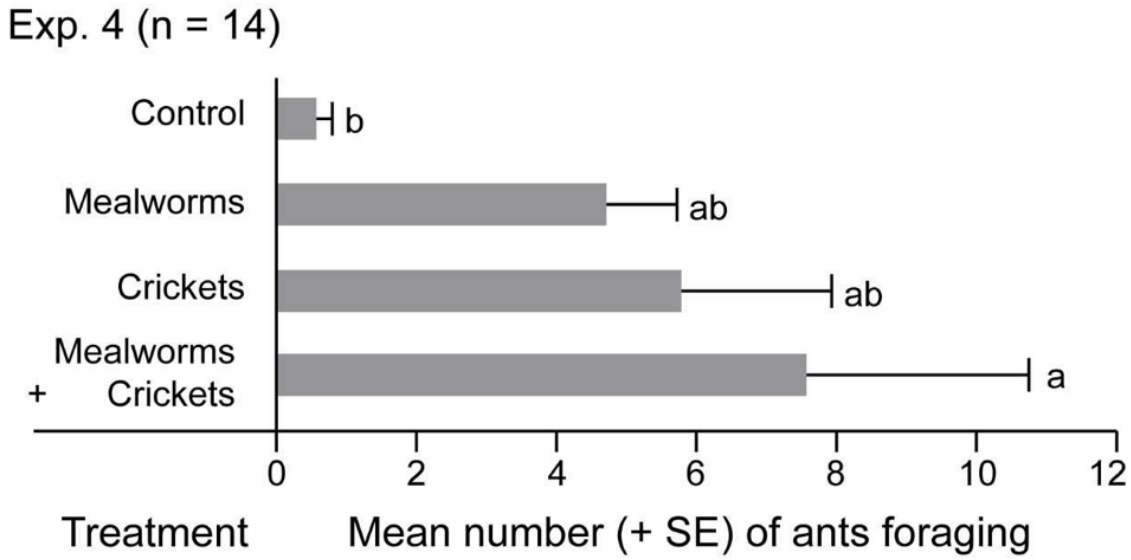

Figure 2.4. Mean numbers (+ SE) of European fire ants, *Myrmica rubra*, present on test stimuli (2-g equivalents) in experiment 4. Bars with different letters indicate statistically different foraging responses by ants (Mixed effect analysis using nest as a random effect and treatment as a fixed effect, with Tukey-Kramer pairwise comparisons of means; $P < 0.05$).

(O4) Experiments 5-7: Effect of freeze-drying baits on lab-foraging responses of EFAs

In laboratory arena bioassays (Fig. 2.1, B), bait treatment had a significant effect on foraging responses of EFAs in each of experiments 5-7 (Exp. 5: $F_{3,15} = 5.04$, $P = 0.004$; Exp. 6: $F_{3,20} = 13.91$, $P < 0.0001$; Exp. 7: $F_{3,19} = 16.89$, $P < 0.0001$; Fig. 2.5). Crickets, apples, or apples and crickets in combination rehydrated after freeze-drying were as effective as corresponding fresh baits, and more effective than corresponding freeze-dried (but not rehydrated) baits which did not differ from unbaited controls (Fig. 2.5).

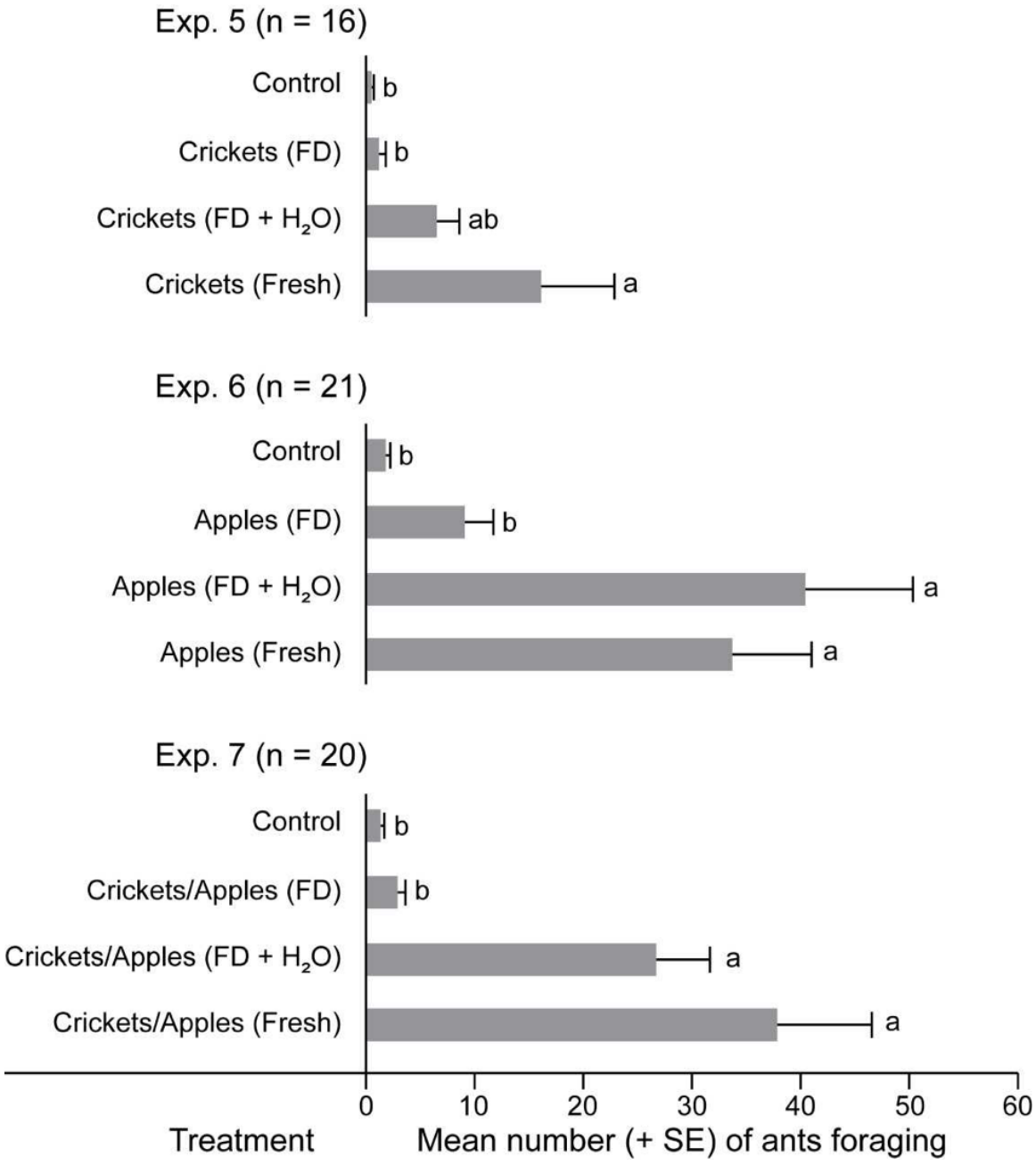

Figure 2.5. **Mean numbers (+ SE) of European fire ants, *Myrmica rubra*, present on test stimuli (2-g equivalents) in experiments 5-7. Stimuli were presented fresh, freeze-dried (FD) or freeze-dried and rehydrated (H_2O). In each experiment, bars with different letters indicate statistically different foraging by ants (Mixed effect analysis using nest as a random effect and treatment as a fixed effect, with Tukey-Kramer pairwise comparisons of means; $P < 0.05$).**

(O5) Experiment 8: Comparative effect of freeze-drying or heat-drying combined cricket and apple baits on lab-foraging responses of EFAs

In laboratory arena bioassays (Fig. 2.1, B), freeze-dried and rehydrated apple/cricket baits attracted 2.5-times more ants than heat-dried and rehydrated apple/cricket baits ($F_{1,19} = 8.68$, $P = 0.0083$; Fig. 2.6).

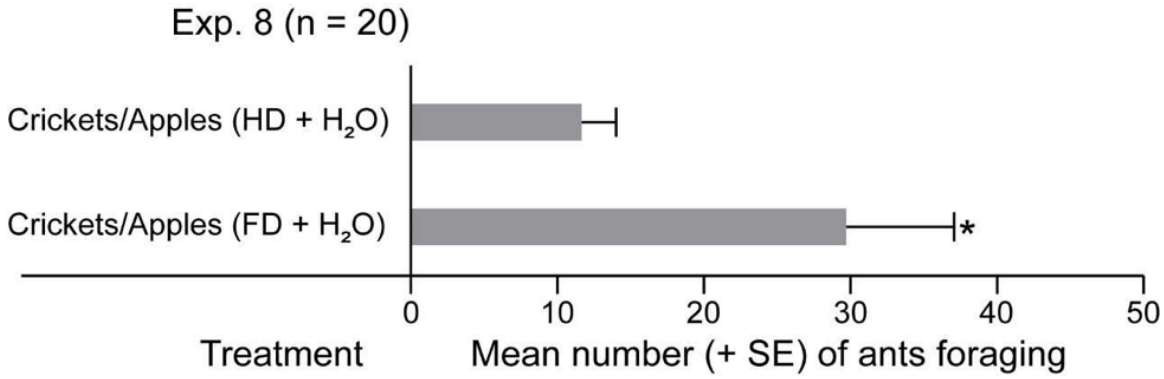

Figure 2.6. Mean numbers (+ SE) of European fire ants, *Myrmica rubra*, present on test stimuli (2-g equivalents) in experiments 8. Stimuli (apples/crickets) were heat-dried (HD) or freeze-dried (FD) and rehydrated (H_2O) for testing. The asterisk (*) indicates a statistically significant preference for freeze-dried and rehydrated apples/crickets (Mixed effect analysis using nest as a random effect and treatment as a fixed effect, with Tukey-Kramer pairwise comparisons of means; $P < 0.05$).

Discussion

Our objective was to develop a food bait for EFAs that meets the three major requirements for commercial development and operational implementation in ant control: (1) strong appeal to foraging ants, (2) cost-effective industrial production, and (3) extended shelf life.

We studied the foraging response of ants to food baits, assuming that EFAs, like many other ant species (Raubenheimer & Simpson, 1999; Dussutour & Simpson, 2008), require a diverse and balanced diet comprising carbohydrates, proteins and lipids. Prior to testing for any interactive effects between macronutrients such as carbohydrates and proteins, we wanted to explore the effect of select sugar and protein sources on foraging responses of EFAs. Although aphid honeydew is readily consumed by foraging EFAs (Naumann and Higgins 2015) we discounted it as an unobtainable sugar source for ant

baits. As EFAs readily accept alternate sugar sources such as sliced apples (R. Higgins; pers. comm.), we offered ants in a field experiment apples, oranges and bananas as potential sugar sources. Similar responses of EFAs to these fruits (Fig. 2.2, top) may have been prompted by the same natural sugars (e.g., fructose, glucose, sucrose) that are present not only in aphid honeydew (Fischer et al., 2005) but also in these fruits (Blüthgen & Fiedler, 2004; Novgorodova, 2019). Unlike these carbohydrate sources, the protein/lipid sources (tuna, pollen, seeds, mealworms) that we field-tested triggered differential foraging responses by ants (number of ants recruited to baits), with mealworms – as a surrogate for dead insects – eliciting the strongest response (Fig. 2.2, bottom). The complex and rich nutritional composition of mealworms (Zhao et al., 2016) is likely the major contributing factor for the ants' preferential foraging response. Insects, in general, as a food source contain more crude protein (40-75% of dry weight) than conventional meat such as beef or poultry. Insects are also rich in saturated and unsaturated fatty acids, offer a larger number of essential and semi-essential amino acids, and even provide vitamins and micronutrients (Tang et al. 2019, and cited references therein).

The nutritional value of insects as a dietary source for ants is also reflected in the reproductive strategy of myrmecochorous plants. These plants secure the seed dispersal service of ants by offering rewards in the form of seeds with lipid-rich appendages (elaiosomes). Compared to seeds, elaiosomes contain more easily digestible compounds such as amino acids and monosaccharides (Fischer et al., 2008), and thus have nutritional value to ants not unlike that of dead or live prey insects (Hughes et al., 1994). Blending mealworms with apples further increased the amount of readily digestible monosaccharides and, as a result, made the apple/mealworm blend more appealing to ants than apples or mealworms alone (Fig. 2.3).

Affordability of an insect control technology is a major determinant for its adoption by end-users. This notion also applies to the concept of lethal food baits for EFAs. Bait affordability is primarily affected by bait ingredient and manufacturing costs. When it became apparent that EFAs forage more readily on mealworms as a protein and lipid source than on tuna, pollen and seeds (Fig. 2.2, bottom), we investigated more affordable alternatives to mealworms as potential bait constituents. As crickets are already industrially produced for human consumption (https:www.fastcompany.com), we compared foraging responses of EFAs to crickets, mealworms and to both combined

(Fig. 2.4), and demonstrated that crickets could replace mealworms as a more affordable insect source in ant baits. However, it is conceivable that there are even cheaper alternatives to crickets. For example, there is ever increasing interest in using black soldier flies, *Hermetia illucens* L., not only for recycling agricultural waste products and household food scraps, but also as a protein source for aquaculture, animal feed, pet food and human consumption (Rumpold et al., 2013). As soldier flies have a short generation time and larvae feed on "surplus" decomposing organic material, production costs for soldier fly pupae as bait constituents should be minimal.

Cost savings for food bait ingredients may also be realized by substituting apples with select mono- or di-saccharides, such as glucose, fructose or sucrose, provided that EFAs can use them as sugar sources. The carpenter ant *Camponotus pennsylvanicus* (DeGeer), e.g., readily accepts sucrose (Cannon, 1998) but black garden ants, *Lasius niger* L., which forage heavily on aphid honeydew, preferentially seek sugar solutions containing the honeydew-specific (and more expensive) tri-saccharide melezitose (Völkl et al. 1999; Detrain et al., 2010). Incorporating select mono- or di-saccharides in lieu of apples as bait constituents would eliminate the need for drying apples and thereby reduce bait manufacturing costs.

The shelf life of food baits is linked to their water content. Apples contain up to 86% water (https://quadram.ac.uk/spotlight/apple-facts; https://fdc.nal.usda.gov/fdc-app.html#/food-details/171688/nutrients) which in ant baits would facilitate microbial metabolism of nutrients and food degradation (Ramirez-Mares et al., 1995; Soliva-Fortuny et al., 2004). Therefore, freeze- or heat-drying apples and insects as bait constituents would be mandatory to achieve the desired extended shelf life of baits. Freeze-dried apples and crickets, carefully rehydrated, were as appealing to foraging ants as fresh apples and crickets (Fig. 2.5), indicating that freeze-drying retained most of the bait's "ant appeal". Similarly, when we compared the foraging responses of EFAs to apple/cricket baits that were either heat- or freeze-dried prior to rehydration, the ants significantly preferred the freeze-dried bait (Fig. 2.6).

The combined challenges of minimizing bait manufacturing costs and achieving prolonged shelf life could be addressed by adding paraben preservatives to moist baits which suppress microbial growth (Kleinfeld & Ellis, 1967; Eklund, 1980). Alternatively, most if not all macronutrients of an ultimate EFA food bait could be select mono- or di-

saccharides and essential amino acids which as solids do not readily spoil over prolonged periods of time. The development of this type of bait, however, would require careful testing of the various mono- and di-saccharides as well as amino acids that are preferentially sought by foraging EFAs. Prior to deployment, dry baits could be dissolved in water and offered as liquid baits which are readily taken up by ants (e.g., Greenberg & Klotz, 2000). Any bait composition, however, must be compatible with the insecticidal agent.

In conclusion, food baits most appealing to foraging EFAs ought to comprise sources of carbohydrates and dead insects, which provide proteins, lipids and vitamins. Costs for bait ingredients could be kept minimal by selecting insects as bait ingredients that are already mass-produced for animal feed or human consumption, and by substituting apples with select mono- or disaccharides provided they are readily accepted by EFAs. Freeze-drying and rehydrating baits prior to deployment would afford the desired shelf life and retain bait effectiveness but would incur significant costs for freeze-drying. These costs could potentially be avoided by manufacturing moist baits preserved with parabens. Alternatively, all bait ingredients could consist of proven-effective mono- and di-saccharides as well as essential amino acids which are solids and in dry form should have extended shelf life. They could also be dissolved and stored in water but these liquid baits – once opened – should not be stored for extended periods of time to avoid bait spoilage.

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Chapter 3. Effects of trail pheromone purity, dose, and type of placement on recruiting European fire ants, *Myrmica rubra,* to food baits*

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Chapter Abstract

Trail pheromones of ants guide nest mates to a food source. Applications of synthetic trail pheromone guiding ants to poisoned food baits may expedite the demise of nests and help control invasive ant species. The trail pheromone of the invasive European fire ant (EFA), *Myrmica rubra* Linnaeus (Hymenoptera: Formicidae), has previously been identified as 3-ethyl-2,5-dimethylpyrazine. To facilitate its development as an operational EFA control tactic, our objectives were to determine the effects of (1) pheromone purity (isometrically pure or isomeric mixture), (2) pheromone dose [2, 20, 200, 2,000 ant equivalents (AEs)], and (3) type of pheromone placement (pheromone surrounding a food bait or leading toward it) on ant recruitment to baits. In laboratory binary choice experiments, pheromonal purity was not essential for the ants' recruitment response. The pheromone dose of 200 AEs elicited the strongest recruitment of ants. Pheromone applied in a circle surrounding a food bait, instead of a line leading toward the food bait, was ineffective in recruiting ants, suggesting that 3-ethyl-2,5-dimethylpyrazine has a guiding but not an attractive function to EFAs.

Introduction

Many ant species use a trail pheromone to coordinate foraging efforts (Billen and Morgan 1998). When a foraging ant finds a food source, she returns to the nest depositing trail pheromone. Nestmates then use this pheromone trail to find their way to the food source, reinforcing the trail in the process. Foragers of some ant species may deposit more pheromone, and thus recruit more nestmates, when they have found a high-quality food source (de Biseau et al. 1991; Czaczkes et al. 2015). Essentially,

nestmates make collective decisions about the food sources they want to exploit. Trail pheromone-guided foraging is common in ants, including the European fire ant (EFA), *Myrmica rubra* Linnaeus (Hymenoptera: Formicidae) (Cammaerts-Tricot 1973).

In their native range in Europe, EFA nests typically consist of a few hundred workers and several queens (Fokuhl et al. 2007). They prey on small invertebrates and tend to aphids, from which they collect honeydew. They also collect elaiosomes and disperse seeds (Fokuhl et al. 2007). In their invaded range (the East and West coasts of North America), EFAs do not engage in nuptial flights (Groden and Drummond 2005) where winged queens mate with males and then land elsewhere to start a new nest. Instead, new nests occur by “budding” (queens and workers leaving the original nest and establishing a new nest nearby), without nuptial flight. As a result, local population densities of EFAs can become so high that they greatly reduce native ant species (Naumann and Higgins 2015). As EFAs swarm and sting aggressively when disturbed, they can render gardens, lawns and parks unusable (Garnas 2004; Saltman 2016).

EFAs are efficient foragers, apparently locating resources faster than other ant species (Garnas 2014). To coordinate foraging efforts, EFAs use a trail pheromone (3-ethyl-2,5-dimethylpyrazine) which they release from the poison gland (Evershed et al. 1982). Individual ants follow trails of synthetic pheromone (Evershed et al. 1981) but it is not yet known whether synthetic pheromone can be exploited for EFA control.

Current EFA control methods are sparse. Excavating nests and spraying them with permethrins is labor-intensive and may need to be repeated if one or more queens have been missed in the process (Higgins 2017). Topical insecticide treatments of nests are ineffective as they kill only those ants who happen to be above ground at the time of treatment. The concept of lethal food baits to control EFAs is appealing because EFAs forage socially and, through trophallaxis, might effectively distribute any lethal agent among nestmates (Brian and Abbott 1977). The efficacy of lethal food baits likely hinges upon attractive food odorants and/or synthetic trail pheromone to guide foraging ants to food baits.

The EFA trail pheromone component 3-ethyl-2,5-dimethylpyrazine is commercially available (Acros Organics, Part of Thermo Fisher Scientific, New Jersey, USA) as a mixture of two isomers: 2-ethyl-3,5-dimethyl pyrazine and 2-ethyl-3,6

dimethylpyrazine (the latter synonymous with 3-ethyl-2,5-dimethyl pyrazine). This isomeric mixture is inexpensive and thus suitable for development as an EFA trail pheromone lure. However, as non-natural pheromone isomers can interfere with optimal behavioral responses of insects (Roelofs and Comeau 1971), it is important to determine whether pure and isomeric 2-ethyl-3,5-dimethylpyrazine elicit comparable trail-following behavior by EFAs.

Responses of insects to natural or synthetic pheromone are typically dose-dependent. Larger amounts of synthetic pheromone as trap lures often result in greater trap captures of target insects (Collignon et al. 2019). However, there are exceptions. In many ant species, the concentration of trail pheromone modulates the trail-following response of nestmates (Evershed et al. 1982; Kohl et al. 2001, 2003; Morgan et al. 2006) with the highest pheromone concentration not always eliciting the strongest response. Workers of the Western carpenter ant, Camponotus modoc Wheeler (Hymenoptera: Formicidae), follow a low-dose synthetic pheromone trail for a longer distance than they follow a high-dose synthetic pheromone trail (Reynard et al. 2019). Moreover, leafcutter ants, *Atta sexdens sexdens* Linnaeus (Hymenoptera: Formicidae), walk longer distances on low-dose pheromone trails than on high-dose pheromone trails (Morgan et al. 2006). Dose-dependent responses to synthetic trail pheromone have also been studied with EFAs (Evershed et al. 1982) but only in the absence of a food source.

A lethal food source must be deployed together with synthetic trail pheromone to achieve the demise of EFA nests. The placement method of synthetic trail pheromone likely determines its effectiveness for recruitment of ants to lethal food baits. In nature, trail pheromone is deposited in a line to guide nestmates toward a food source (Cammaerts-Tricot 1978). The deployment of synthetic pheromone trails in an analogous manner seems challenging. This challenge likely prompted an alternative way of pheromone placement. By simply adding trail pheromone directly to lethal baits, bait consumption by Argentine ants, *Linepithema humile* Mayr (Hymenoptera: Formicidae), increased and thus resulted in greater ant mortality and lower ant activity in the field (Greenberg and Klotz 2000; Welzel and Choe 2016). However, the recruitment effect of this type of pheromone placement may be dependent upon both the volatility of the pheromone and the propensity of foraging ants to be attracted to, rather than guided by, trail pheromones.

Our overall objective was to determine whether the synthetic trail pheromone of EFAs (3-ethyl-2,5-dimethylpyrazine) can be deployed to increase recruitment of nestmates to food baits. Our specific objectives were to determine the effects of (1) pheromone purity (isometrically pure or isomeric mixture), (2) pheromone dose (2-2,000 ant equivalents), and (3) type of pheromone placement (pheromone surrounding a food bait or leading toward it) on ant recruitment to baits.

Materials and Methods

Colony Collections

We collected EFA colonies in the spring and summer of 2016-2018 from Inter River Park (North Vancouver, BC), the Burnaby and Regional Allotment Garden (Burnaby, BC), and the VanDusen Botanical Garden (Vancouver, BC). To locate nests, we walked in a transect while disturbing the soil by shuffling our feet. As EFAs respond quickly when disturbed, it is easy to locate a nest entrance. Wearing nitrile gloves to protect ourselves from stings, we excavated circa 30 cm^3 of soil surrounding a nest entrance, and placed it in a large bucket (19 L, 38 cm tall × 30 cm diam.) We slowly sifted through this soil by hand, collecting about 10 queens, 200-300 workers, and 50-100 larvae/pupae from each nest. We transferred these ants to artificial nest housings (see below) in our laboratory.

Rearing of Experimental Ants

We kept ant colonies indoors in the Science Research Annex (49° 16'33" N, 122° 54'55" W) of Simon Fraser University at a temperature of 25° C and a photoperiod of 12L:12D. The rearing protocol took into account that colonies need both an enclosed nest housing (hereinafter referred to as the “nest-box”) and a surrounding foraging arena to exhibit normal behavior (Drees and Ellison 2002) (Fig. 3.1 A). The nest-box consisted of a small plastic container (15 × 15 × 9 cm), two-thirds of which filled with potting soil (Sunshine® Mix #4, Sungro, Agawam, MA, USA). A 10-cm^2 hole in nest-box lids was covered with plastic mesh (Lumite Saran fabric, 10 ml) to allow for ventilation and water misting (see below). Each nest-box was placed inside a foraging arena, consisting of a plastic tote (41 × 29 × 24 cm or 58 × 43 × 31 cm) fitted with a mesh-covered hole (10 × 10 cm) in the lid to allow air flow. Ants entered and exited the nest-box through a 15-cm

long Nalgene tubing (3.175 mm diam; Nalgene 180 PVC non-toxic autoclavable Lab/FDA/USB V1 grade; Thermo Scientific, Waltham, MA, USA). The 5-cm wide upper-most rim of each foraging arena was coated with a 1:1 mixture of paraffin oil (White, Anachemia, Lachine (Montreal), Que, Ca) and petroleum jelly (Vaseline) to prevent ants from escaping.

Twice per week, we added sources of protein (canned tuna, cat food, dog food, tofu, canned beans, canned chicken, dehydrated shrimp, anchovy paste, dead mealworms, dead blowflies, dead crickets, sunflower seeds, pumpkin seeds, luncheon meat, corned beef), and carbohydrates (canned oranges, apple slices, apple sauce, grapes, candy, honey, sugar water, cranberry sauce, raisins) to the foraging arena, thus allowing ants to leave their nest-box and forage (Fig. 3.1 A). We provided such a large variety of foods to minimize the possibility that ants "learned" to forage for a specific food type. We provided water in a test tube fitted with a piece of cotton which we replaced whenever it became moldy or dry. Twice per week, we rehydrated nest-boxes by spraying water through the mesh window.

General design of binary choice bioassay

We deprived colonies of food (but not water) 5-7 days prior to bioassays, testing each colony only once for each stimulus. We ran experimental replicates during the ants' photoperiod in six circular arenas (122 cm diam. × 40 cm height) housed in a dedicated bioassay room (22-23 ºC) (Fig. 3.1 B). To initiate a bioassay, we temporarily closed the entrance tube to a nest-box with a piece of cotton. We then removed this nest-box from the foraging arena (Fig. 3.1 A) and placed it in a circular arena, such that the nest entrance tube was perpendicular to two strips of filter paper (each 30 × 3 cm) taped to the arena floor. To provide a food source for foraging ants, we placed a mixture of macerated apples and mealworms (1:1 ratio; 2 g total) on top of a circular piece of damp cotton (9 cm diam) at the distal end of each strip. After the ants (in their transferred nest-box) had acclimatized in the bioassay arena for 10 min, we treated – by random assignment – paper strips with a 25-µl aliquot of either synthetic trail pheromone dissolved in pentane or a pentane control. Immediately following the application of test stimuli, we opened the nest-box entrance to initiate the bioassay. We terminated all experimental replicates after 2 h (when ant foraging activity peaked according to preliminary tests) at which time we counted the number of ants present on food baits or

cotton circles. We then collected all foraging ants by aspirator or hand and returned them together with the nest-box to their original foraging arena. We tested pheromone in ant equivalents (AEs), with a mean pheromone amount of 5.8 ng occurring in a single worker ant (Cammaerts et al. 1981).

Specific Experiments

Table 3.1. List of research objectives (O) and stimuli tested in experiments 1-3.

Experiment #	Test stimuli (T)	Replicates
O_1: Determine the effect of pheromone isomer		
1	T_1: pure pheromone[a]; T_2: pheromone mixture[b] (200 AEs[c] tested for both T_1 and T_2)	14
O_2: Determine the optimal dose of pheromone[d]		
2	T_1: 2 AEs; T_2: Solvent control T_1: 20 AEs; T_2: Solvent control T_1: 200 AEs; T_2: Solvent control T_1: 2000 AEs; T_2: Solvent control	20 20 20 15
O_3: Determine the effect of pheromone[d] placement		
3	T_1: Pheromone circle around bait (200 AEs); T_2: Solvent control circle around bait	20

[a]3-ethyl-2,5-dimethylpyrazine;

[b]mixture of 3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine at a 1:1 ratio;

[c]AE = ant equivalent of trail pheromone (5 ng)

[d]tested as pheromone mixture (see b)

Effect of 3-ethyl-2,5-dimethylpyrazine alone and in combination with isomeric 2-ethyl-3,5-dimethyl pyrazine on trail-following responses of ants (Exp. 1)

The commercial source of the EFA trail pheromone component 3-ethyl-2,5-dimethylpyrazine (Acros Organics, Part of Thermo Fisher Scientific, NJ, USA) is an isomeric mixture of 3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethyl pyrazine at a 1:1 ratio. To isolate the natural (EFA-produced) isomer, we employed a high-performance liquid chromatograph (HPLC) (Waters Corporation, Milford, MA, USA) fitted with a Synergy Hydro Reverse Phase C18 column (250 mm × 4.6 mm, 4 µ; Phenomenex, Torrance CA, USA) and operated by a HPLC System (600 Controller, 2487 Dual Absorbance Detector, Delta 600 Pump). Eluting the isomeric mixture with a 0.75-$mL^{-1\ min}$ flow of acetonitrile separated the two isomers but without baseline

resolution. By collecting only the second half of the later eluting target isomer (3-ethyl-2,5-dimethylpyrazine) peak, we could obtain material for bioassays with 83 to 93% purity.

To determine whether the non-natural isomer (2-ethyl-3,5-dimethylpyrazine) in the isomeric mixture had any adverse effect on trail-following responses of EFAs, we used the general two-choice bioassay design described above (Fig. 3.1 B), and tested the isolated synthetic trail pheromone component 3-ethyl-2,5-dimethylpyrazine alone [200 AEs (1,000 ng per trail)] *versus* the isomeric mixture containing 3-ethyl-2,5-dimethylpyrazine at the same amount.

Effect of trail pheromone dose on trail-following responses of ants (Exp. 2)

To determine the trail pheromone dose that elicits the strongest trail-following responses by EFAs, we tested each of four doses of synthetic trail pheromone (10, 100, 1000, 10000 ng, equivalent to 2, 20, 200 and 2000 AEs) dissolved in pentane (25 µL) versus a pentane control (25 µL) (Table 1). We applied the pheromone treatment stimulus and the solvent control stimulus in 30-cm long streaks on two non-overlapping paper strips (each 30 × 3 cm) secured in a straight line to the bioassay arena floor (see general experimental design) (Fig. 3.1 B).

Effect of trail pheromone placement on ant recruitment to baits (Exp. 3)

To determine whether EFAs respond to trail pheromone applied around a food source, rather than leading toward it (Exps. 1,2), we modified the experimental design of experiments 1 and 2 in that we surrounded each of the two food baits with a circular strip of filter paper (15 cm diam; 2 cm wide; cut from a circular filter paper) (Fig. 3.1 C) and treated one strip with synthetic trail pheromone (isomer mixture, 200 ng) and the other with a solvent control.

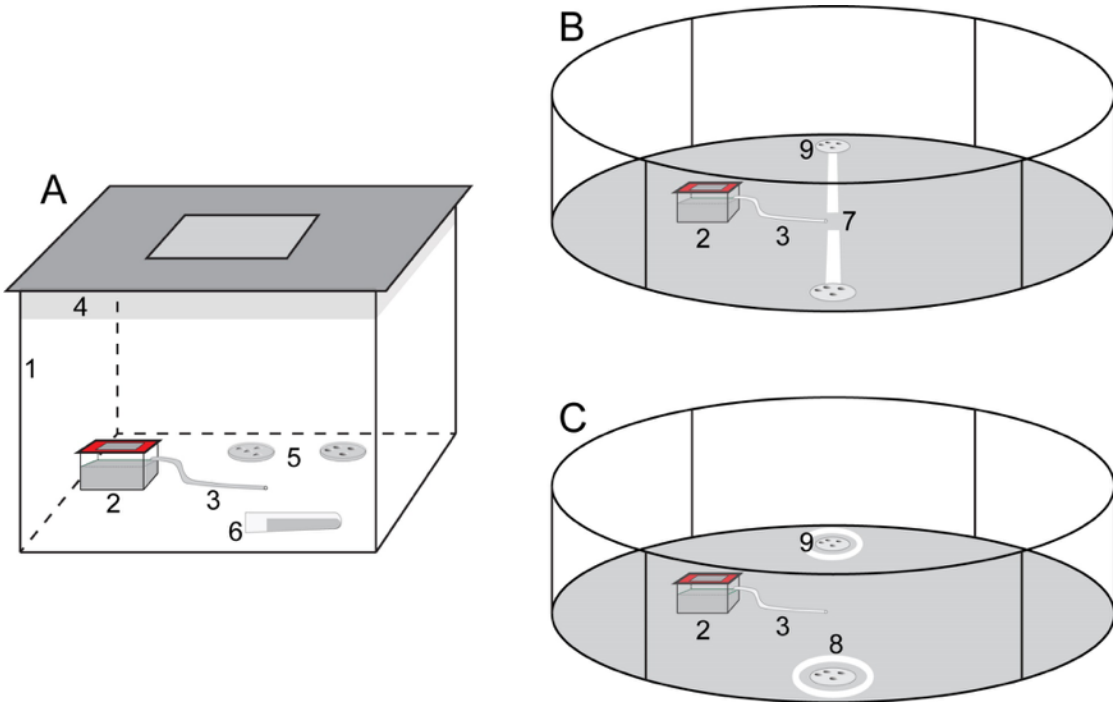

Figure 3.1. **Graphical illustrations of: (A) the set-up for maintaining ant colonies in the insectary annex, consisting of a foraging arena (41 × 58 × 31 cm) (1) which housed the ants' nest box (15 × 15 × 9 cm) (2) fitted with a Nalgene tubing (3.1 mm diam, 15 cm long) (3) for nest entry and exit. The upper 5 cm of the foraging arena was lined with a slippery mixture of petroleum jelly and mineral oil (4). The nest was provisioned with sources of food and water presented in Petri dishes (5) and in form of a moist cotton plug confining a water reservoir inside a test tube (6), respectively; (B,C) the experimental design for testing the effect of synthetic trail pheromone on foraging decisions by ants. For each replicate in design (B), the nest box (2) was placed inside a large circular bioassay arena such that the entry and exit tubing (3) was perpendicular to two filter paper strips (30 × 3 cm) (6), each leading to a circular piece of damp cotton (7 cm diam) (7) with a food bait; in design (C), each food bait was surrounded by a circular filter paper strip (15 cm diam, 2 cm wide), one of which treated with synthetic trail pheromone and the other with a solvent control.**

Statistical analyses

We analyzed the data of experiment 1 (effect of pure and isomeric pheromone on trail-following responses of ants) with a t-test in JMP. We analyzed the data of experiment 2 (effect of trail pheromone dose on trail-following responses of ants) using a general linear mixed model with a binomial distribution and a logit link function using the GLIMMIX procedure in SAS. The response variable was the proportion of ants

responding to the pheromone bait out of the total number of ants responding. Ant equivalents were a fixed effect, and the site of nest origin was a random effect. The over-dispersion in the model was accounted for in the model by scaling the standard errors proportional to the deviance. Post-hoc tests were used to determine differences in pairs of mean responses using a Tukey-Kramer adjustment. We analyzed data of experiment 3 (effect of trail pheromone placement) using a t-test in JMP.

Results

Effect of 3-ethyl-2,5-dimethylpyrazine alone and in combination with isomeric 2-ethyl-3,5-dimethylpyrazine on trial pheromone responses of ants (Exp. 1)

There was no difference in the proportion of ants that were recruited to a food bait by the trail pheromone 3-ethyl-2,5-dimethylpyrazine alone or in combination with isomeric 2-ethyl-3,5-dimethylpyrazine (t = 1.96, n = 13, df = 12, p = 0.07; Fig. 3.2)

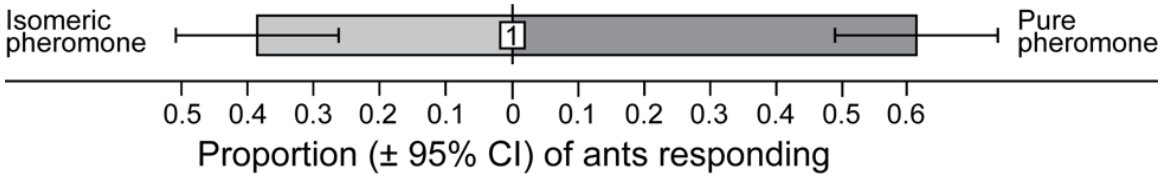

Figure 3.2. Mean proportion of European fire ants present in binary choice arena bioassays (see Fig. 1B) on cotton pads with food bait in response to pure pheromone (3-ethyl-2,5-dimethylpyrazine) or isomeric pheromone (3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine) applied to the paper strip leading to the food bait; t-test, p = 0.07; the number in the bar center (1) represents the one replicate where the nest was not responding (total number of replicates run: n = 14).

Effect of trail pheromone dose on trail-following responses of ants (Exp. 2)

Trail pheromone dose did affect the recruitment response of ants ($F_{3,65} = 11.15$, $p < 0.0001$). When trail pheromone was tested at 2 AEs, it recruited 61% of the foraging ants to the corresponding food bait (n = 20, t = 3.27, p = 0.002; Fig. 3.3). Trail pheromone tested at 20 and 200 AEs recruited 57% and 69% of foraging ants, respectively (20 AEs: n = 19, t = 2.09, p = 0.04; 200 AEs: n = 20, t = 5.99, p < 0.001). At the high dose of 2,000 AEs, the effect decreased to 59% of foraging ants (n = 11, t = 2.27, p = 0.03). The recruitment effect of the 200-AE dose exceeded that of the other

pheromone doses tested (200 vs 2: t = 0.002, p = 0.01; 200 vs 20: t = -5.06, p < 0.0001; 200 vs 2,000: t = 4.34, p = 0.0003; Tukey Kramer analyses).

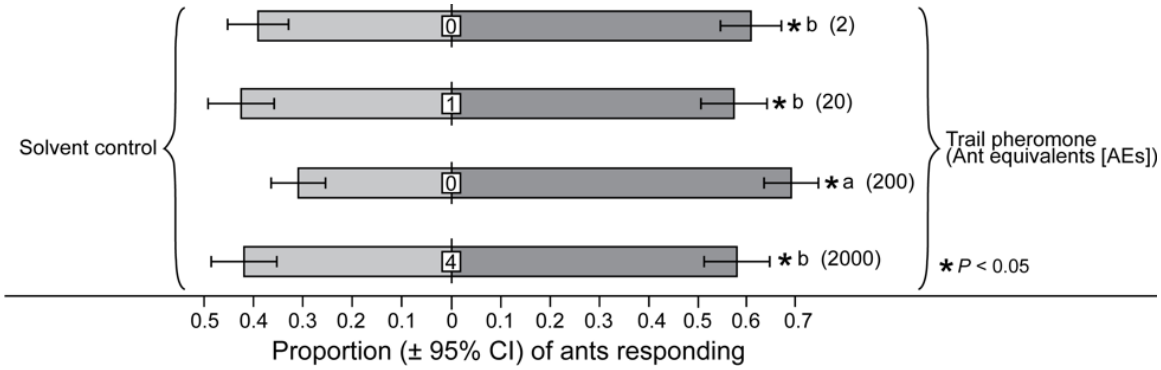

Figure 3.3. Mean proportion of European fire ants present in binary choice arena bioassays (Fig. 1b) on cotton pads with food bait in response to isomeric pheromone (3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine) applied at 2, 20, 200 or 2,000 ant equivalents (AEs; 1 AE = 5 ng of 3-ethyl-2,5-dimethylpyrazine) to the paper strip leading to the food bait, each pheromone dose tested *versus* a solvent control. An asterisk (*) indicates a significant preference for the pheromone stimulus. General linear mixed model, p < 0.05; the 200 AE trail pheromone dose was more effective than all others in recruiting ants to the food bait (Tukey Kramer test adjusted for multiple comparisons, p < 0.05); the numbers in bar centers represent the number of replicates where the nest was not responding; total number of replicates run: n = 20 for each of 2, 20 and 200 AEs; n = 15 for 2000 AEs.

Effect of trail pheromone placement on ant recruitment to baits (Exp. 3)

When trail pheromone was applied at the most effective dose (200 AEs) on a filter paper strip encircling the food bait (Fig. 3.1 C), instead of leading toward it (Fig. 3.1 B), the pheromone failed to recruit ants to the food bait (t = 0.76, n = 19, df = 18, p = 0.47; Fig. 3.4), indicating that the pheromone has no attractive properties.

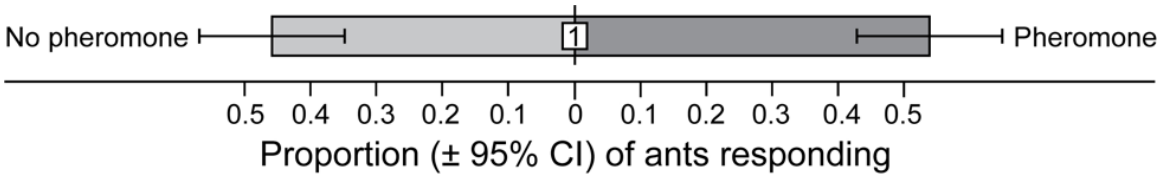

Figure 3.4. Mean proportion of European fire ants present in binary choice arena bioassays on food baits surrounded by a circular filter paper strip which was treated, or not (control), with synthetic trail pheromone (1,000 ng of a synthetic mixture of 3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine) (Fig. 1C). The trail pheromone near the food bait had no effect on recruitment responses of ants; t-test, p > 0.05; the number in the bar center (1) represents the one replicate where the nest was not responding (total number of replicates run: n = 20).

Discussion

Our data provides helpful information for the development of synthetic trail pheromone as a means for guiding foraging EFAs to lethal food baits. Trail pheromone-guided rapid location of food by foraging ants and transport to the nest, coupled with food-sharing trophallaxis, will facilitate the demise of nests and help control local EFA populations. Our study has addressed important questions about pheromone purity, as well as optimal dose and type of pheromone placements that needed to be answered prior to operational pheromone implementation.

Affordability of pheromone-based control tactics is a key determinant for their development and sustained use. Pheromone-based pest control tactics may be more earth-friendly, but due to their species-specificity are typically more expensive than conventional insecticides that control a wide range of pest insects. Low pheromone synthesis costs contribute to the affordability of pheromone-based control tactics and can sometimes be achieved by producing a mixture of optical or structural isomers rather than stereospecifically pure pheromone. The commercially available, and relatively affordable, source of the EFA trail pheromone contains not only the trail pheromone 3-ethyl-2,5-dimethypyrazine but also 2-ethyl-3,5-dimethypyrazine as a non-pheromonal structural isomer. The presence of optical or structural isomers in pheromone lures is known to sometimes interfere with the optimal effectiveness of the pheromone. For example, the attractiveness of synthetic (+)-disparlure, the sex pheromone of the gypsy moth, *Lymantria dispar* (Bierl *et al*. 1970)*,* is reduced in the presence of its antipode (-)-disparlure in a racemic pheromone lure (Miller *et al.* 1977).

Similarly, tetradecenyl acetates with a double bond near C11 added to the sex pheromone (*Z*)-11-tetradecenyl acetate of the red-banded leaf roller, *Argyrotaenia velutinana*, greatly decreases pheromonal attraction of male moths (Roelofs and Comeau 1971). In light of these findings, it was important to determine whether a non-pheromonal isomer impurity (2-ethyl-3,5-dimethypyrazine) in the commercial source of the EFA trail pheromone had any adverse effect on trail-following responses of EFAs. As both pure and isomerically impure synthetic trail pheromone prompted similar trail following responses by EFAs (Fig. 3.2), it is now possible to use isomerically impure pheromone for operational development.

The amount of trail pheromone deposited by ants, or experimentally applied, affects the trail following response of nest mates, as shown in carpenter ants, *Camponotus spp*. (Kohl *et al*. 2001, 2003; Renyard *et al*. 2019), the leaf cutting ant *Atta sexdens sexdens* (Morgan *et al*. 2006), and the EFA (Evershed *et al*. 1982; this study). In our study, trail following responses were pheromone dose-dependent. As little as 2 AEs of trail pheromone (0.33 ng/cm) were sufficient to enhance recruitment of EFAs to food baits (Fig. 3.3), but a dose of 200 AEs (33 ng/cm) was more effective. The effect was still present but decreased with the highest dose (2,000 AEs or 330 ng/cm) (Fig. 3.3). These data differ from a previous report (Evershed *et al*. 1982) that a trail pheromone dose of only 0.0319 ng/cm triggered the strongest trail following responses. These differences are not that surprising given that pheromone behavior in ants is very context-dependent (Vander Meer and Alonso 1998). Evershed *et al*. (1982) presented a circular trail to groups of 25 or 50 EFA workers in the absence of a food bait, recording the ants' responses for 15 min. We, in contrast, offered an entire nest [at least 100 EFA workers per nest; 15-20 nests (see Table 1)] a choice between two paper strips treated with either a solvent control or the EFA trail pheromone, each strip leading to a food bait where we counted the number of recruited ants 2 h after bioassay initiation. The decreased activity of the highest trail pheromone dose (2,000 AEs) may reflect the behavioral choices of ants to ignore seemingly overcrowded trails that don't allow for efficient foraging (Dussutour *et al*. 2004; Burd *et al*. 2011). It also may be a result of sensory overload, an effect that has been used to disrupt foraging behavior in the Argentine ant (Suckling *et al*. 2011; Sunamura *et al*. 2011).

Trail pheromones of ants may embody some (Möglich *et al*. 1974) or all (Vander Meer *et al*. 1990) of the following functions: orientation induction (prompting trail

following of nest mates), orientation (guiding foragers along trails), and short-range attraction (attracting foragers to trails). These functions may be mediated by a single-component pheromone or a multiple component pheromone blend (Jackson *et al*. 1990). To test whether the single-component trail pheromone of EFAs not only has a guiding function, but also an attractive function, we deployed either the trail pheromone or a solvent control on a circular paper strip surrounding a food bait, each bait 30 cm away from the nest entrance. The very similar numbers of ants recruited to these two food baits (Fig. 3.4), provided evidence that the EFA trail pheromone does not function as an attractant, despite being highly volatile (Evershed et al.1982). Keeping a low profile by using a trail pheromone without attractive function may be advantageous in settings of high nest density, where ants could otherwise readily eavesdrop on their neighbors' pheromone trails and exploit them.

Our experiments were not designed to explore whether the EFA trail pheromone has an orientation induction function (Vander Meer *et al*. 1990), prompting or initiating trail following by nestmates. Based on prior literature (Cammaerts-Tricot 1978) it seems that a component of the Dufour's gland may serve this function.

Future research will need to determine the efficacy of synthetic trail pheromone in field settings and explore potential types of pheromone formulations (e.g., pheromone-laden ropes) and modes of deployment, all coupled with lethal food baits.

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Chapter 4. Ants Sense, and Follow, Trail Pheromones of Ant Community Members*

*A near identical version of this chapter has been published: Jaime Chalissery, Asim Renyard, Regine Gries, Danielle Hoefele, Santosh K. Alamsetti, Gerhard Gries (2019) Insects 10:383; doi:10.3390/ insects10110383; JC, AR, DH & GG conceived the study; RG & JC ran electrophysiological recordings; SKA synthesized trail pheromones; RG prepared pheromone blends & JC ran behavioral bioassays; JC & AR graphed and analyzed data; JC, AR & GG wrote the first draft; all authors reviewed and approved of the final draft for submission.

Chapter Abstract

Ants deposit trail pheromones that guide nestmates to food sources. We tested the hypotheses that ant community members (Western carpenter ants, *Camponotus modoc*; black garden ants, *Lasius niger*; European fire ants, *Myrmica rubra*) (1) sense, and follow, each other's trail pheromones, and (2) fail to recognize trail pheromones of allopatric ants (pavement ants, *Tetramorium caespitum*; desert harvester ants, *Novomessor albisetosus*; Argentine ants, *Linepithema humilis*). In gas chromatographic-electroantennographic detection analyses of a six-species synthetic trail pheromone blend (6-TPB), *La. niger*, *Ca. modoc*, and *M. rubra* sensed the trail pheromones of all community members and unexpectedly that of *T. caespitum*. Except for *La. niger*, all species did not recognize the trail pheromones of *N. albisetosus* and *Li. humilis*. In bioassays, *La. niger* workers followed the 6-TPB trail for longer distances than their own trail pheromone, indicating an additive effect of con- and hetero-specific pheromones on trail-following. Moreover, *Ca. modoc* workers followed the 6-TPB and their own trail pheromones for similar distances, indicating no adverse effects of heterospecific pheromones on trail-following. Our data show that ant community members eavesdrop on each other's trail pheromones, and that multiple pheromones can be combined in a lure that guides multiple species of pest ants to lethal food baits.

Introduction

Ant colonies use multimodal communication signals to coordinate specific tasks such as foraging, nest defense, and cooperative brood care (Hölldobler and Wilson 1990). Trail pheromone signals are particularly important in the context of foraging

(Morgan 2009). When a forager has located a profitable food source and then returns to her nest, she deposits trail pheromones that guide nest mates to the same resource (Morgan 2009). Additional foragers recruited to this resource may also deposit trail pheromones and thus reinforce the original trail (Morgan 2009; Detrain and Deneubourg 2008), effectively resulting in collective decisions by nestmates as to which resource to exploit (Detrain and Deneubourg 2008; Beckers et al 1990).

Pheromone trails leading to persistent food sources are generally well maintained by foragers (Morgan 2009) and thus are readily exploited by (heterospecific) non-nestmates (Hölldobler and Wilson 1990; Vander Meer and Alonso 1998) that learn about the location of profitable food sources through eavesdropping (Wilson 1965; Adams 1990; Gobin et al. 1998; Menzel et al. 2010). We use the term “eavesdropping” here to describe the behavior of ants gleaning trail pheromone information from community members but not to imply inevitably adverse effects for any community member involved. Indeed, aggressive encounters of ants with non-nest mates on shared (eavesdropped) trails (Hölldobler and Wilson, 1990; Gobin et al. 1998; Menzel et al. 2010) are kept to a minimum, in part, by using dissimilar foraging schedules. Temporal partitioning of activity schedules has been reported for workers of *Ca. pennsylvanicus* and *Formica subsericea* that forage on the same aphid-infested trees but at different times of the day (Klotz 1984), and for workers of *Ca. beebei* that follow trails of *Az. charifex* when *Azteca* ants are resting (Hölldobler and Wilson, 1990; Wilson 1965; Gobin et al. 1998; Menzel et al. 2010). We anticipate that mutual recognition of pheromone trails is more likely for co-evolved ant species than for native and invasive species. However, two exceptions are conceivable. First, the invasion event took place a long time ago and, over time, the invading species has become a well-established and integrated community member. Second, the invading species is closely related to native species and thus produces a similar trail pheromone.

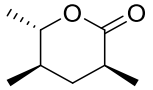
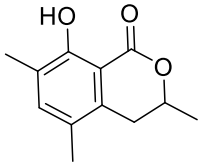
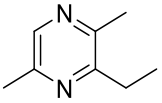
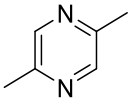
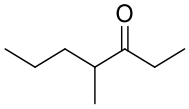
Ant communities in the Lower Mainland of British Columbia (BC), Canada are complex and comprise both native and invasive species. For the purpose of this study, we have selected three species that co-exist in the same community: (1) the Western carpenter ant, *Camponotus modoc* (Formicinae), which is considered native to the Pacific Northwest and has been recorded in British Columbia as early as 1919 (Buckell 1932); (2) the black garden ant, *Lasius niger* (Formicinae), which is native to Europe, and possibly to North America, having been recorded in the New World as early as 1979

(Buckell 1932; Higgins and Lindgren 2012; Naumann 1999); and (3) the European fire ant, *Myrmica rubra* (Myrmicinae), which is native to Europe but has invaded the Pacific Northwest and other parts of North America, likely in the first decade of the 20th century (Groden et al. 2005; Naumann and Higgins 2015). While *Ca. modoc* and *La. niger* have co-existed for at least 39 years (Naumann 1999), *M. rubra* as a more recent adventive is known to have occurred in ant communities of British Columbia's Lower Mainland for nearly 20 years (Naumann and Higgins 2015) and has already become a well-established and integrated community member. During 20 years of co-existence, all three species might have "learned" to sense each other's trail pheromones.

We prepared the trail pheromone components currently known for these three species (*Ca. modoc*: (2S,4R,5S)-2,4-dimethyl-5-hexanolide (henceforth "hexanolide") (Renyard et al. 2019); *La. niger*: 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (henceforth "isocoumarin") (Bestmann et al. 1992); *M. rubra*: 3-ethyl-2,5-dimethylpyrazine (Evershed et al. 1982) in a synthetic blend (Table 1). This blend also contained 3-ethyl-2,6-dimethyl pyrazine (a non-natural isomer in the commercial source of the *M. rubra* trail pheromone). To determine whether *Ca. modoc*, *La. niger*, and *M. rubra* sense the trail pheromones not only of community members but also of allopatric ant species, we expanded the synthetic blend to include the trail pheromone of the pavement ant, *Tetramorium caespitum* (2,5-dimethylpyrazine), the desert harvester ant, *Novomessor albisetosus* (4-methyl-3-heptanone), and the Argentine ant, *Linepithema humilis* ((Z)-9-hexadecenal) (Attygalle and Morgan 1983; Hölldobler et al 1995; Van Vorhis 1982).

Here, we tested the hypotheses that sympatric *Ca. modoc*, *La. niger*, and *M. rubr*a (1) sense, and behaviorally respond to, each other's trail pheromones, and (2) fail to recognize the trail pheromones of allopatric ant species (*T. caespitum*, *N. albisetosus*, *Li. humilis*).

Table 4.1. List of trail pheromones (and select species producing them) comprising the six-trail pheromone blend (6-TPB) tested in circular trail bioassays (Figure 4.1) and in electrophysiological recordings (Figure 4.2, Table 4.2). In trail bioassays (Figures 4.3 – 4.5), the trail-following of *Camponotus modoc*, *Lasius niger*, and *Myrmica rubra* was each tested in response to (*i*) the 6-TPB formulated in pentane, (*ii*) their own trail pheromone formulated in pentane, and (*iii*) a pentane control. Stimuli were tested at 1–2 ant equivalents (AE)/58 µL (*Ca. modoc*) and 1–2 AEs/25 µL (*La. niger* and *M. rubra*) to account for the length differential of stimulus trails that were tested for large ants (*Ca. modoc*) and small ants (*La. niger* and *M. rubra*) (see Methods for detail).

Study Species	Name of Pheromone (Amount Tested; Ant Equivalents (AEs))	Pheromone Structures (Synthetic Sources a–e)
Ca. modoc	(2*S*,4*R*,5*S*)-2,4-Dimethyl-5-hexanolide (7.5 ng; 2 AEs) ("hexanolide") (Renyard et al. 2019)	 (a)
La. niger	3,4-Dihydro-8-hydroxy-3-7-trimethylisocoumarin (0.5 ng; 1 AE) ("isocoumarin") (Bestmann et al. 1992)	 (a)
M. rubra	3-Ethyl-2,5-dimethylpyrazine (5 ng; 1 AE) (Evershed et al. 1982)	 (b)
T. caespitum	2,5-Dimethylpyrazine (1 ng; 1 AE) (Attygalle and Morgan 1983)	 (c)
N. cockerelli	4-Methyl-3-heptanone (10 ng;1 AE) (Hölldobler et al. 1995)	 (d)
Li. humilis	(*Z*)-9-Hexadecenal (10 ng; 1 AE) (Van Vorhis 1982)	CH_3-$(CH_2)_4$-CH=CH-$(CH_2)_7$-CHO (e)

(a) Synthesized as described by Renyard et al. 2019; (b) Acros Organics, New Jersey, USA (contains 3-ethyl-2,5-dimethylpyrazine at 50%); (c) Aldrich Chem Co. Milwaukee, WI, USA; (d) oxidized from 4-methyl-3-heptanol (Sigma-Aldrich, St. Louis, MO, USA); (e) oxidized from (*Z*)-9-hexadecenol (Sigma-Aldrich).

Materials and Methods

Experimental Insects

Lasius niger

Between 13 and 31 August (2018), 5–10 ants were collected in various containers from each of 20 sites throughout Vancouver and Burnaby, British Columbia. Ants were bioassayed within 24 h of collection, and then cold-euthanized for taxonomic confirmation using multiple keys (Naumann et al. 1999; Mackay and Mackay 2002; Wilson 1955; Wing 1968).

Camponotus modoc

Collection and maintenance of *Ca. modoc* nests have recently been described in detail (Renyard et al. 2019). Briefly, infested log sections were kept in large plastic bins (64 cm × 79 cm × 117 cm) in an outdoor undercover area exposed to natural light and temperature cycles throughout the year. Each plastic bin housing a nest was connected via clear PVC tubing (2.54 cm I.D., Nalgene™ 180; Sigma-Aldrich, St. Louis, MO, USA) to a glass aquarium (51 × 28 × 30 cm), which served as the ants' foraging area provisioned with blow flies, live mealworms, honey, apples, canned chicken, and 20% sugar water, all *ad libitum*.

Myrmica rubra

In the spring and summer of 2017 and 2018, 20 nests of *M. rubra* were dug out of the ground at Inter River Park (North Vancouver, BC, Canada), the Regional Allotment Garden (Burnaby, BC, Canada), and the VanDusen Botanical Garden (Vancouver, BC, Canada). Nests were kept indoors in the Science Research Annex of Simon Fraser University (49°16′33″ N, 122°54′55″ W) at 25 °C and a photoperiod of 12 h L to 12 h D. Nests were housed in small Tupperware dishes (15 × 15 × 9 cm) (Rubbermaid®, Newell Brands, Atlanta, GA, USA & Royal Sponge Manufacturing Ltd., Toronto, ON, Canada), which were fitted with sterilized potting soil as nesting material and placed inside a small or large tote (41 × 29 × 24 cm; 58 × 43 × 31 cm) that served as the ants' foraging area. Twice a week, the nests were sprayed with water and provisioned with food (fruits, nuts, mealworms, and processed meat). Test tube water reservoirs were replaced when low.

Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analyses of Synthetic Ant Trail Pheromones

For GC-EAD analyses and behavioral bioassays, a synthetic blend of six ant trail pheromones (see above), henceforth six-trail pheromone blend (6-TPB; Table 4.1), was prepared. The blend was analyzed by gas chromatographic-electroantennographic detection (GC-EAD), with procedures and equipment previously described in detail [25,26]. Briefly, the GC-EAD setup employed a Hewlett-Packard 5890 gas chromatograph (GC) fitted with a DB-5 GC column (30 m × 0.32 mm I.D.; J&W Scientific, Folsom, CA, USA). Helium served as the carrier gas (35 $cm \cdot s^{-1}$) with the following temperature program: 50 °C for 1 min, 20 $°C \cdot min^{-1}$ to 280 °C. The injector port and flame ionization detector (FID) were set to 260 °C and 280 °C, respectively. For GC-EAD recordings (three antennae each for *Ca. modoc*, *La. niger*, and *M. rubra*), an antenna was carefully dislodged from a worker ant and suspended between two glass capillary electrodes (1.0 × 0.58 × 100 mm; A-M Systems, Carlsborg, WA, USA) prepared to accommodate the antenna and filled with a saline solution (Staddon and Everton 1980).

General Design of Trail-Following Bioassays

All bioassays were run within a metal scaffold (123 × 57 × 36 cm) encased in black fabric to eliminate external visual stimuli, lit from above with two fluorescent lights (48″ 32 W F32T8, one plant and aquarium bulb, and one daylight bulb, Phillips, Amsterdam, The Netherlands), and fitted with a video camera (Sony HDR CX210, Sony, Tokyo, Japan or Canon FS100 A, Canon, Tokyo, Japan) mounted above the bioassay arena (Figure 4.1). The edges of the bioassay arenas (see below) were coated with a mixture of petroleum jelly and mineral oil to prevent the escape of bioassay ants.

The specific experimental design to test trail-following responses accounted for body size differentials of large ants (*Ca. modoc*) and small ants (*La. niger* and *M. rubra*). The design for testing *Ca. modoc* was previously described (Renyard et al. 2019) and is outlined here. *Camponotus modoc* was tested in a large plexiglass arena (64 × 44 × 10 cm) fitted with a filter paper (18.5 cm diam; Sigma-Aldrich, St. Louis, MO, USA), with its circular circumference marked with pencil in 1 cm intervals (58 marks total) and treated with one of three test stimuli (see below) at 1–2 ant equivalents (AE)/58 µL. Each

bioassay ant (n = 60) entered the arena by exiting a 15 mL Falcon™ “holding” tube (Thermo Fisher Scientific, Waltham, MA, USA) through a hole cut in its tapered tip.

Lasius niger and *M. rubra* were tested in a Pyrex petri dish (15 cm diam) fitted with a small circular filter paper (9.0 cm diam), with its circumference marked with pencil in 1 cm intervals (25 total) and treated with one of three test stimuli (see below) at 1–2 AEs/25 µL. Each worker ant of *La. niger* (n = 60) and *M. rubra* (n = 60) entered the Petri dish by exiting a 1.5 mL Axygen™ MaxyClear Snaplock “holding” microtube (Thermo Fisher Scientific, Waltham, MA, USA) through a hole cut in its tapered tip. Bioassays of large and small ants were initiated by removing the cotton plug from the exit hole of a holding tube and were terminated after 5 min (*Ca. modoc*) and 10 min (*La. niger* and *M. rubra*). Filter papers were prepared for bioassays by applying a continuous trail of test stimulus [(*i*) synthetic 6-TPB; (*ii*) synthetic trail pheromone of the bioassay ant; or (*iii*) a solvent control; Table 4.1].

The number of 1 cm intervals an ant had followed during a bioassay served as the response criterion and was analyzed by viewing the video footage. Ants not leaving their holding tube after 10 min were considered non-responders and excluded from analyses. Between bioassays, all preparative surfaces and bioassay arenas were cleaned with 70% EtOH and hexane, and the experiment room was aired out for 5 to 10 min by opening an exterior door. A new ant was tested for each treatment. All *La. niger* and *M. rubra* ants were collected from different laboratory or field colonies. Worker ants of *Ca. modoc* were collected from six colonies maintained in an outdoor enclosure.

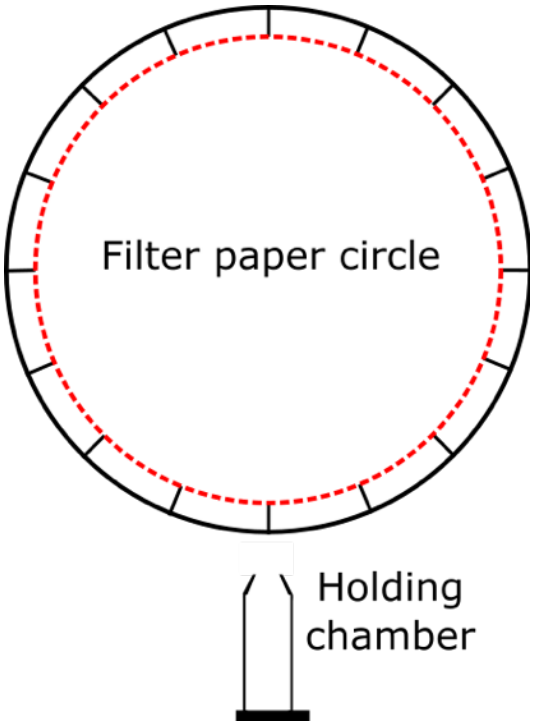


Figure 4.1. **Graphical illustration of the experimental design used for testing trail-following of Western carpenter ants, *Camponotus modoc*, black garden ants, *Lasius niger*, and European fire ants, *Myrmica rubra*, in response to their own trail pheromone or a complex blend of six trail pheromones (see Table 4.1). To account for body size differentials of large ants (*Ca. modoc*) and small ants (*La. niger*; *M. rubra*), bioassay arenas were large (64 cm wide × 44 cm long × 10 cm high) or small (circular, 15 cm diam × 1 cm high), and the diameter of the filter paper was 18.5 and 9 cm, respectively. Pheromone trails were applied to the filter paper along the red dotted line (which was absent in bioassays).**

Statistics

R (Version 3.5.0; multicomp, plotrix, & plyr packages) was used to analyze the data and produce graphics (Hothorn et al. 2008; Lemon and Plotrix 2006; Wickham 2011). A generalized linear model (GLM; quasi-Poisson distribution) was used to analyze the distances ants travelled following trails in response to the various types of trails presented. An analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test were used to determine significant differences in mean distance travelled in response to trail type.

Results

Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analyses of Synthetic Ant Trail Pheromones

In GC-EAD analyses, *La. niger* antennae responded to (in the order of elution) synthetic 2,5-dimethylpyrazine, 4-methyl-3-heptanone, 3-ethyl-2,5-dimethylpyrazine, 3-ethyl-2,6-dimethyl pyrazine, hexanolide, isocoumarin (its own trail pheromone), and (Z)-9-hexadecenal (Figure 4.2; Table 4.2). Antennae of *Ca. modoc* responded to 2,5-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 3-ethyl-2,6-dimethyl pyrazine, hexanolide (its own trail pheromone), and isocoumarin (Figure 4.2; Table 4.2). Antennae of *M. rubra* responded to 2,5-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine (its own trail pheromone), 3-ethyl-2,6-dimethyl pyrazine, hexanolide, and isocoumarin (Figure 4.2; Table 4.2).

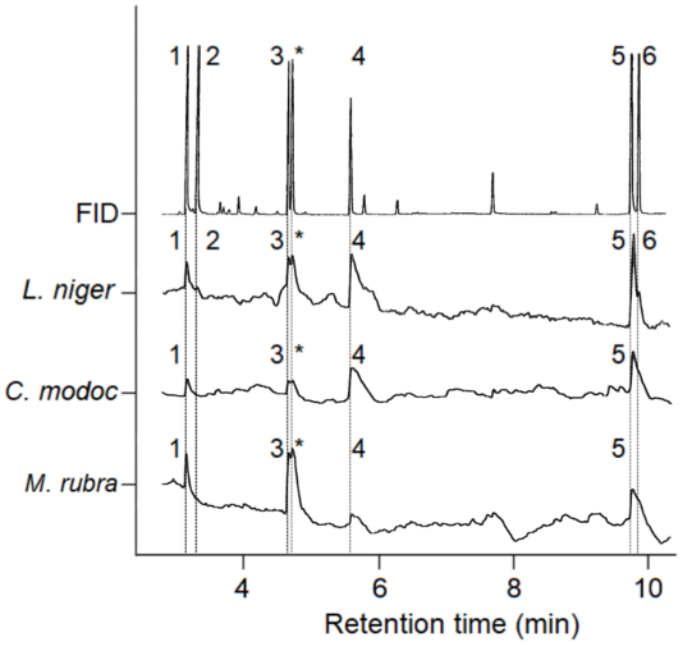


Figure 4.2. **Representative recordings (n = 3 each) of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: Antenna of a *Lasius niger*, *Camponotus modoc*, or *Myrmica rubra* worker ant) to pheromone components present in the six-trail pheromone blend (see Table 4.1). Numbers in the FID trace refer to the following pheromone components: (1) 2,5-dimethylpyrazine; (2) 4-methyl-3-heptanone; (3) 3-ethyl-2,5-dimethylpyrazine; * = 3-ethyl-2,6-dimethyl pyrazine (non-natural isomer present in synthetic source); (4) (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide; (5) 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin; and (6) (*Z*)-9-hexadecanal.**

Table 4.2. List of trail pheromone components produced by sympatric ant species inhabiting ant communities in the Pacific Northwest (Western carpenter ants, *Camponotus modoc*; black garden ants, *Lasius niger*; European fire ants, *Myrmica rubra*) and by allopatric ant species (pavement ants, *Tetramorium caespitum*; desert harvester ants, *Novomessor albisetosus*; Argentine ants, *Linepithema humilis*), as well as information as to whether community members (*La. niger*, *Ca. modoc,* and *M. rubra*) antennally respond to these components in electrophysiological recordings (summary of gas chromatographic-electroantennographic detection (GC-EAD) results; see Figure 4.2).

Distribution	Species	Trail Pheromone	Produced by/Antennal Response		
			La. niger	*Ca. modoc*	*M. rubra*
Sympatric	*La. niger*	Isocoumarin *	yes/yes	yes/yes	no/yes
	Ca. modoc	Hexanolide **	no/yes	yes/yes	no/yes
	M. rubra	3-Ethyl-2,5-dimethylpyrazine	no/yes	no/yes	yes/yes
Allopatric	*T. caespitum*	2,5-Dimethylpyrazine	no/yes	no/yes	no/yes
	N. albisetosus	4-Methyl-3-heptanone	no/yes	no/no	no/no
	Li. humilis	(*Z*)-9-Hexadecenal	no/yes	no/no	no/no

* 3,4-Dihydro-8-hydroxy-3,5,7-trimethylisocoumarin; ** 2,4-Dimethyl-5-hexanolide.

Trail-Following Bioassays

There were significant differences in the distances (mean ± SE) that worker ants of *La. niger* travelled following trails of the 6-TPB (495.5 ± 57.5 cm), the isocoumarin (199.3 ± 43.1 cm), and the solvent control (32.2 ± 14.0 cm) (ANOVA, $F = 34.028$, degrees of freedom (df) = 2, residual df = 57, $p < 0.001$; Figure 4.3). Based on Tukey's HSD tests, all distances differed from one another (pairwise comparisons: Isocoumarin *vs.* solvent control: $p = 0.001$; 6-TPB *vs.* solvent control: $p < 0.001$; 6-TPB *vs.* isocoumarin: $p < 0.001$).

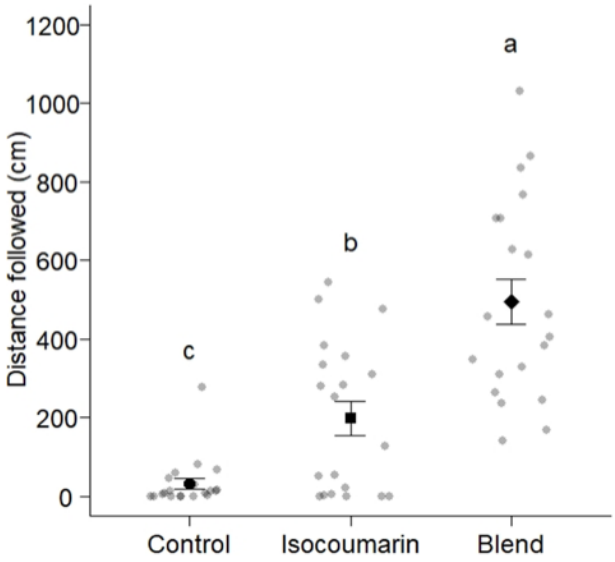


Figure 4.3. Distances worker ants of *Lasius niger* (n = 60) travelled following trails of synthetic 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (the known trail pheromone of *La. niger* [17]), a six-trail pheromone blend (Table 4.1), and a solvent control applied to the circumference of a circular filter paper (diam: 90 mm) marked in 1-cm intervals (Figure 4.1). Grey and black symbols show the distance that each ant and 20 ants on average (mean ± whiskers), respectively, travelled following trails. Means associated with different letters are statistically different (Tukey's honest significant difference (HSD) test, $p < 0.01$); six out of 66 ants tested did not enter the bioassay arena and were excluded from this data set.

There were also significant differences in the distances that worker ants of *Ca. modoc* travelled following trails of the 6-TPB (213.1 ± 48.7 cm), the hexanolide (206.0 ± 32.7 cm), and the solvent control (69.1 ± 12.6 cm) (ANOVA, F = 7.5583, df = 2, residual df = 57, $p < 0.01$; Figure 4.4). Based on Tukey's HSD tests, distances were different between the 6-TPB and the solvent control ($p < 0.01$), and between the hexanolide and the solvent control ($p < 0.01$), but were statistically the same between the 6-TPB and the hexanolide ($p = 0.99$).

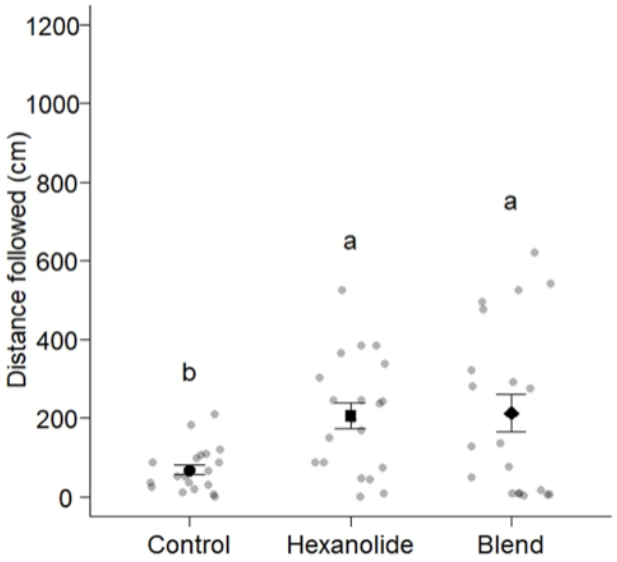


Figure 4.4. Distances worker ants of *Camponotus modoc* (n = 60) travelled following trails of synthetic (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide (the known trail pheromone of *Ca. modoc* [16]), a six-trail-pheromone blend (Table 4.1), and a solvent control applied to the circumference of a circular filter paper (diam: 185 mm) marked in 1 cm intervals (Figure 4.1). Grey and black symbols show the distance that each ant and 20 ants on average (mean ± whiskers) travelled, respectively, following trails. Means associated with different letters are statistically different (Tukey's honest significant difference (HSD) test, $p < 0.01$); two out of 62 ants tested did not enter the bioassay arena and were excluded from this data set.

There were no significant differences in the distances that worker ants of M. rubra travelled following trails of the 6-TPB (22.9 ± 8.7 cm), the 3-ethyl-2,5-dimethylpyrazine (41.2 ± 8.3 cm), and the solvent control (32.9 ± 8.1 cm) (ANOVA, F = 1.1555, df = 2, residual df = 57, p = 0.3222; Figure 4.5).

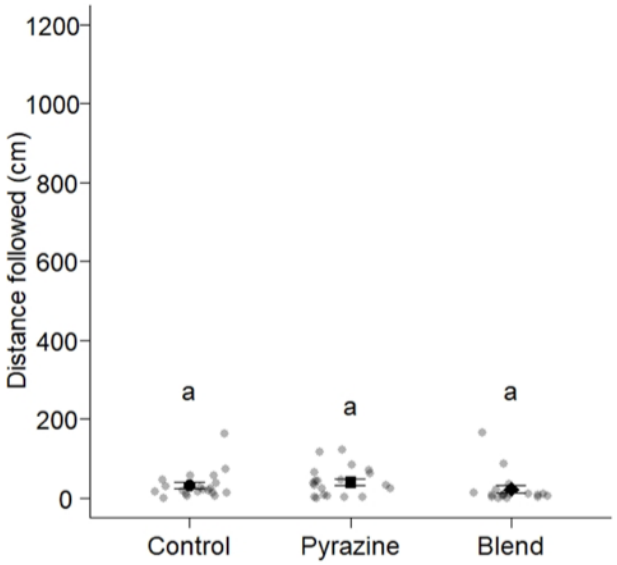


Figure 4.5. Distances worker ants of *Myrmica rubra* (n = 60) travelled following trails of synthetic 3-ethyl-2,5-dimethylpyrazine (the known trail pheromone of *M. rubra* [19]), a six-trail-pheromone blend (Table 4.1), and a solvent control applied to the circumference of a circular filter paper (diam: 90 mm) marked in 1-cm intervals (Figure 4.1). Grey and black symbols show the distance that each ant and 20 ants on average (mean ± whiskers) travelled, respectively, following trails. Means associated with different letters are statistically different (Tukey's honest significant difference (HSD) test, $p < 0.01$).

Discussion

As predicted, *La. niger*, *Ca. modoc*, and *M. rubra* did sense (antennally respond to) the trail pheromone of all community members (*La. niger*, *Ca. modoc*, *M. rubra*; Figure 4.2) and, except for *La. niger*, did not recognize the trail pheromones of two allopatric ant species (*N. cockerelli* and *Li. humilis*; Table 4.2). That all three ant species sensed the trail pheromone of allopatric *T. caespitum* could be due its molecular structure (2,5-dimethylpyrazine) resembling that of the *M. rubra* trail pheromone (3-ethyl-2,5-dimethylpyrazine). In light of our behavioral data that the 6-TPB (which contains trail pheromones of con- and hetero-specifics) readily induced trail-following behavior of *Ca. modoc* and *La. niger*, it seems that these ants either simply ignore (*Ca. modoc*), or indeed eavesdrop on (*La. niger*), each other's trail pheromone communication. In general, eavesdropping ants can face aggression, increased competition, or even displacement (Hölldobler and Wilson, 1990; Wilson 1965; Gobin et al. 1998; Menzel et al. 2010; Klotz 1984) but in the ant community we studied here, eavesdropping may accrue more benefits than harm, or at least, no harm. This inference is based on our

bioassay data showing that (*i*) *La. niger* workers followed trails of the 6-TPB for a longer distance than they followed their own trail pheromone (isocoumarin), and (*ii*) *Ca. modoc* workers followed trails of the 6-TPB and their own pheromone (hexanolide) for similar distances.

Unexpectedly, workers of *M. rubra* followed trails of the 6-TPB and their own trail pheromone (3-ethyl-2,5-dimethylpyrazine) only as much as a solvent control trail, demonstrating no effect of the trail pheromone in this type of bioassay. There are at least two explanations why *M. rubra* did not follow a trail of synthetic 3-ethyl-2,5-dimethylpyrazine. Prior studies that demonstrated distinct trail following by *M. rubra*, either in “no-choice bioassays” comparable to our experimental design (Evershed et al. 1982) or in “binary-choice arena bioassays” (Hoefele et al. 2020), tested the responses of multiple workers (the entire nest), whereas we tested the responses of individual ants. Given the small foraging range and high nest density of *M. rubra* in North America (Hoefele et al. 2020; Higgins, pers. comm), it is conceivable that nest mates do not forage on their own but engage in group foraging, as shown in many *Myrmica* species (Fedoseeva 2015). Group foraging entails cooperative interactions, where, for example, a successful forager recruits nestmates and physically guides them to the food source (Fedoseeva 2015). Group foraging may improve the overall foraging effort of a nest and facilitate transport of food particles that are too heavy for single ants to carry (Carroll and Janzen 1973). Alternatively, the trail pheromone blend of *M. rubra* comprises not only 3-ethyl-2,5-dimethylpyrazine but additional pheromone components, which, thus far, have eluded identification.

The evidence presented here that some ant community members eavesdrop on and exploit each other’s trail pheromone has major implications for ant control. Food baits laced with lethal agents show promise as an ant control tactic because many ants share food through trophallaxis and thus may spread the poison together with the food throughout their entire nest. The effect of lethal food baits can be enhanced by adding attractants. For example, the admixture of trail pheromone to food baits increased bait consumption by the invasive Argentine ant, *L. humile* (Welzel and Choe 2016). In *M. rubra*, a path of synthetic trail pheromone leading from a nest to a food bait is more effective in recruiting foragers than applying the trail pheromone around a food bait (Hoefele et al. 2020).

Commercial development of trail pheromones for ant control is contingent upon economic feasibility. With so many important ant species in need of control, and with each species producing its own trail pheromone, manufacturing species-specific (single target) trail pheromone lures (ropes, strings) does not seem economically viable. However, if trail pheromones of multiple ant species were to be combined in a single lure (multiple targets), with potential synergism and no antagonism between components, as shown in our study, then an ant control tactic that couples a lethal food bait with a trail pheromone lure seems commercially feasible. As an added advantage, the ant species targeted for control would not even need to be identified by a pest control professional or the lay person buying the control technology in a retail store.

Future studies should aim to strengthen the proof of concept presented in our study. Trail pheromones of major ant pests such as the red imported fire ant, *Solenopsis invicta*, should be added to the multiple-species trail pheromone lure and tested for the response of *S. invicta* and other species. Moreover, research needs to be initiated on dispensers capable of sustained release of trail pheromones in field experiments and, eventually, operational applications.

Conclusions

All three select members of ant communities in the Lower Mainland of British Columbia (*La. niger, Ca. modoc, M. rubra*) sensed each other's trail pheromone and, except for *La. niger*, did not recognize the trail pheromones of two allopatric ant species (*N. cockerelli and Li. humilis*). Workers of *La. niger* followed a synthetic trail pheromone blend (containing the trail pheromone of all three community members and those of three allopatric ant species) for a longer distance than they followed their own trail pheromone, and *Ca. modoc* workers followed this blend and their own trail pheromone for similar distances. Apparently, these ants either ignore (*Ca. modoc*), or indeed eavesdrop on (*La. niger*), each other's trail pheromone. Eavesdropping ants may accrue benefits by learning about the location of profitable food sources. If synthetic trail pheromones of multiple pest ant species were to be combined in a single (rope-type) lure, with potential synergism and no antagonism between components (as shown in our study), an ant control tactic that presents a lethal food bait together with a trail pheromone lure seems commercially viable.

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