Communication and foraging ecology of ants

by

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Abstract

Ants coordinate group tasks such as nest defense and foraging with their nestmates. My thesis unravels signals and cues underlying these tasks.

In part 1, I investigate how ants coordinate defense. I characterized pheromonal and vibratory alarm signals of carpenter ants, *Camponotus modoc,* using gas chromatography-mass spectrometry, laser Doppler vibrometry, and video recordings. In bioassays, I then tested the ants' responses to synthetic alarm pheromone and playback of vibratory signals. In response to playback of vibratory signals, ants ran rapidly, or froze, but did not approach the vibratory signals. Exposed to alarm pheromone, ants frequently visited the pheromone source. However, concurrently exposed to both alarm pheromone and vibratory signals, ants visited the pheromone source less often but spent more time 'frozen'. The ants' modulated responses to bi-modal signals seem adaptative to avoid predation by avian predators.

In part 2, I investigate how ants locate and assess food sources, and whether acquired knowledge about the ants' nutrient preferences can be applied to curb populations of invasive pest ants. I demonstrate that food sources rich in carbohydrates or proteins prompt long-distance attraction of *C. modoc* worker ants, and that attraction of ants to plant inflorescences is mediated by specific, rather than shared, floral odorants. I show that *C. modoc* workers deposit (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide as their trail pheromone to guide nestmates to food sources. I further show that workers of European fire ants, *Myrmica rubra*, and *C. modoc* discern between mono-, di- and tri-saccharides, and between essential amino acids (EAAs) and non-essential amino acids. In a field experiment, colonies of *C. modoc* and *M. rubra* preferentially consumed EAAs and sucrose, respectively, with no sustained shift in preferred macro-nutrient over the course of the foraging season. Importantly, the presence of a less preferred macro-nutrient in the nutrient bait did not diminish the bait's 'appeal' to foraging ants in diverse ant taxa. In a further field experiment in a public park infested with *M. rubra,* I show that treatment colonies provided with a lethal liquid bait (4.55% sucrose; 1% EAA; 1% boric acid) significantly declined, whereas control colonies provided with the corresponding non-lethal bait did not.

Keywords: Pheromone; vibroacoustic; nutrient consumption; diet choice; pest control

Dedication

I dedicate this thesis to my parents who have provided me unwavering support to achieve this goal.

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Chapter 1.

Introduction

1.1. Ecological roles of ants and their colony life

Ecologically, ants are impactful animals (Wilson, 1987; Peeters and Ito, 2015). They are abundant and present in nearly every terrestrial habitat, serving diverse and significant ecological roles (Wilson and Holldobler, 2005). They are predators, herbivores, scavengers, and nutrient cyclers as well as partners in mutualistic relationships with fungi, honeydew-producing insects, and plants (Hölldobler and Wilson, 1990). In turn, ants are prey for many animals (Feldhaar, 2011), and make up a significant proportion of a predator's diet (Gyug et al., 2014). Ants compete for resources (nesting sites, food) with each other (Parr and Gibb, 2010), raid neighboring colonies, kidnap brood (D'Ettorre and Heinze, 2001), and parasitically infiltrate other ant nests (D'Ettorre and Heinze, 2001). Their activities and nest-building habits contribute to nutrient cycling, advance the decomposition of organic material, and alter soil chemistry (Del Toro et al., 2012). Based on these activities, ants have been referred to as ecosystem engineers because they alter the physical, chemical and biological conditions of the ecosystems they inhabit (Meyer et al., 2011; Sanders and van Veen, 2011; De Almeida et al., 2020). Not all the ants' activities have positive effects on ant community members. Invasive pest ants, in particular, bite and sting humans and other vertebrates, threaten biodiversity, and cause significant economic damage (Angulo et al., 2022; Gruber et al., 2022).

As eusocial species, ants divide reproductive and non-reproductive tasks (Wilson, 1971). Mated queen ants bear the task of producing workers and (eventually) new reproductive nestmates (Hölldobler and Wilson, 1990). Non-reproductive tasks, such as nest-building, defense, foraging, and brood care are performed by sterile workers (Hölldobler and Wilson, 1990). While workers do not reproduce, they accrue reproductive fitness by altruistically helping the passing of shared genes via their queen (Korb and Heinze, 2008). As worker ants age, their task repertoire expands. Young workers serve primarily as nurses, whereas older workers collectively engage in nest-cleaning, defense, and eventually foraging (Gordon, 2018). Cooperative behavior – or

teamwork among nestmates – is a dynamic and heritable trait shaped by the ants' environment (Gordon, 2016, 2018; Walsh et al., 2020).

Workers performing tasks respond to external cues (e.g., food quality, enemy threats) and social communication signals (e.g., alarm pheromone) by integrating them for collective behavior. For example, individual foraging ants gauge a food resource by assessing its nutritional quality (Cornelius et al., 1996; Völkl et al., 1999; Blüthgen and Fiedler, 2004b; Zhou et al., 2015; Sola and Josens, 2016; Csata et al., 2020; Madsen and Offenberg, 2020) and quantity (Mailleux et al., 2000; Bolek et al., 2012), and then they recruit nestmates using pheromonal, tactile, and motor signals to aid resource collection (Dornhaus and Powell, 2010). Behavioural responses of nestmates are 'plastic', being modulated by prior experience and context. For example, foraging black garden ants, *Lasius niger,* that draw on memory (experience) and respond to trail pheromone forage most effectively (Czaczkes et al., 2011, 2015a). Similarly, *Temnothorax rugatulus* ants that sense alarm pheromone flee in foreign terrain but orient toward it near their nest (Sasaki et al., 2014).

In my thesis, I investigate the cues and signals that ants use to coordinate group tasks in two major aspects of colony life: foraging and nest defense.

1.2. Nest defense

Ants defend their nest against predators because it contains food stores, generations of developing brood, and their entire reproductive potential: the queen(s) (Ayasse and Paxton, 2002; Abbot, 2022). Generally, colonies are stationary and thus a rich target for predators, parasites, and competitors (Ayasse and Paxton, 2002; Abbot, 2022) which exert strong selective pressures for colonies to have adequate defenses (Feldhaar, 2011; Abbot, 2022). Individual workers have defensive armaments, such as stings, spines, toxins, and biting mandibles, to fend off enemies (Nouvian and Breed, 2020). At the colony level, nest-building provides stable living conditions and physically obstructs enemies (Dornhaus and Powell, 2010; Nouvian and Breed, 2020). Moreover, weapons become most effective when they are used in coordinated and cooperative defenses (Dornhaus and Powell, 2010; Nouvian and Breed, 2020). Team defenses to threats rely on rapid and effective communication among nestmates (Verheggen et al.,

2010). Ants may also engage in non-aggressive modes of defense, such as fleeing and nest abandonment (Dornhaus and Powell, 2010)

Ants use pheromonal and vibratory signals to coordinate cooperative defenses (Hölldobler and Wilson, 1990; Hunt and Richard, 2013). Some alarm pheromone components may not only alert nestmates but also serve as defensive toxins (Verheggen et al., 2010; Richard and Hunt, 2013). Substrate-borne vibratory alarm signals – which to date have hardly been studied – are produced by stridulating, scraping, and drumming (Golden and Hill, 2016). Both the volatility of alarm pheromones and the rapid propagation of vibratory signals through substrate enable rapid information flow among nestmates (Verheggen et al., 2010; Hunt and Richard, 2013). Responses of ants to mono-modal signals, such as alarm pheromones or alarm vibrations, have been studied but responses of ants to a bi-modal signal complex including both pheromone and vibration have hardly been studied. For example, distressed leafcutter ants stridulate and produce pheromone, with either alarm signal attracting conspecifics (Markl, 1965).

1.3. Foraging

In ants, adequate nutrition is vital for colony growth and functioning (Feldhaar, 2014; Csata and Dussutour, 2019). Macro-nutrients (e.g., carbohydrates, proteins, lipids) and micronutrients (e.g., B vitamins, salts) are important dietary constituents for colony growth and functioning (Porter, 1989; Evans and Pierce, 1995; Feldhaar et al., 2007; Mankowski and Morrell, 2014; Poissonnier et al., 2014). Carbohydrates are an energy source affecting both the activity of workers (Grover et al., 2007; Kaspari et al., 2012; Shik and Silverman, 2013; Wittman et al., 2018) and their longevity (Grover et al., 2007; Cook et al., 2010; Dussutour and Simpson, 2012; Shik and Silverman, 2013; Bazazi et al., 2016; Arganda et al., 2017; Wittman et al., 2018). Proteins, together with carbohydrates, are required for egg production by queens and for brood development (Porter, 1989; Evans and Pierce, 1995; Feldhaar et al., 2007; Grover et al., 2007; Shik and Silverman, 2013; Mankowski and Morrell, 2014). Therefore, foragers face the challenge of satisfying not only their own nutritional requirements but also those of their nestmates (Csata and Dussutour, 2019).

To meet the nutritional requirements of their colonies, forager ants must locate, gauge, and communicate the location of valuable nutrient resources. How ants locate new nutrient resources has rarely been investigated (Knaden and Graham, 2016). Some species use visual cues (Baroni Urbani et al., 1994; Beugnon et al., 2001), whereas others orient towards nutrient odorants (Schatz et al., 2003; Schiestl and Glaser, 2012; Buehlmann et al., 2014; De Vega et al., 2014; Wanjiku et al., 2014; Yusuf et al., 2014; Fischer et al., 2015) but there is little information as to how ants use odorants to discern between different types of resources. Foraging ants gauge the nutritional quality of a resource based on its composite of key macro- and micro-nutrients . They assess the presence and/or concentration of sugar molecules (Cornelius et al., 1996; Völkl et al., 1999; Blüthgen and Fiedler, 2004b; Zhou et al., 2015; Sola and Josens, 2016; Madsen and Offenberg, 2020), amino acids (Lanza, 1991; Lanza et al., 1993; Blüthgen and Fiedler, 2004a; González-Teuber and Heil, 2009; Csata et al., 2020), lipids (Cornelius et al., 1996), salts (Kaspari et al., 2008; Chavarria Pizarro et al., 2012; Hernández et al., 2012; Vieira and Vasconcelos, 2015), and vitamins (Ricks and Vinson, 1970). Whether and to what extent nutrient preferences differ among ant species, and nutrient compositions of resources affect foraging decisions by ants remain largely unknown. Lastly, many ants deposit trail pheromone to recruit nestmates (Czaczkes et al., 2015b) but less than 1% of trail pheromones have been identified.

Comprehensive knowledge about nutritional preferences of ants could inform the composition and development of lethal food baits for control of (invasive) pest ants. Other control tactics such as insecticide sprays or deployment of biological control agents have not been very successful or have undesirable non-target effects (Gentz, 2009; Hoffmann et al., 2016; Suiter et al., 2021), whereas food baits, in some cases, have been successfully deployed to eradicate invasive pest ant populations (Hoffmann et al., 2016). Lethal baits are effective, if foraging ants consistently forage on these baits and spread them through their colony via food-sharing behaviour (trophallaxis) with their nestmates, queen and brood. Food baits that offers nutrients preferentially and consistently consumed by many ants would greatly improve control of pest ants (Hoffmann et al., 2010).

1.4. Model ants

In my thesis, I worked primarily with two ant species as model organisms: the carpenter ant, *Camponotus modoc* (subfamily: Formicinae), and the European fire ant, *Myrmica rubra* (subfamily: Myrmicinae). Working with *C. modoc*, I studied their foraging ecology, trail pheromone, nutrient preferences, mono- and bi-modal alarm communication, and options for their control in urban environments. Working with *M. rubra*, I studied their nutrient preferences and explored options for their eradication in distinct settings such as recreational parks. Lastly, in some of my studies, I included the thatching ants *Formica oreas* and *F. aserva*, and the black garden ant, *Lasius niger* (all subfamily: Formicinae), for taxonomic diversity and comparative analyses. Below, I describe the life history of all four study species.

In the Pacific Northwest, *C. modoc* is a large, wood-dwelling ant that occupies both forest and urban habitats (Hansen and Akre, 1985; Raley and Aubry, 2006). Many species of carpenter ants have a mostly cryptic foraging habit and consume a generalist diet, feeding on floral and extra-floral nectar, seeds, honeydew, arthropod prey, animal droppings and carrion (Youngs and Campbell, 1984; Hansen and Akre, 1985; Sanders and Pang, 1992; Yamamoto and Del-Claro, 2008). Workers of *C. modoc* are generalist foragers that feed on aphid honeydew, arthropod prey and bird droppings (Tilles and Wood, 1982, 1986; Youngs and Campbell, 1984; Hansen and Akre, 1985). How carpenter ants locate new nutrient resources is largely unknown. Similarly, although certain macro and micro-nutrients are essential for ant development, and ants are known to sense them, it is not clear whether carpenter ants discern between, and prefer, certain food sources, and whether any preferences are driven by essential nutrients. Foraging workers of *C. modoc* navigate to food sources, relying upon visual landmarks (David and Wood, 1980; Hansen and Akre, 1985) and on trail pheromone that nestmates release from their hindgut but the trail pheromone components have not yet been identified. In urban habitats, *C. modoc* readily infests human-made wooden structures, accounting for 78% of structural infestations in Washington (Hansen and Akre, 1985).

Camponotus modoc is a great model species for studying (alarm) communication in formicine ants. Formicine ants are taxonomically diverse (>3000 spp.)(Ward et al., 2016) but little is known about their communication systems. Trail or alarm pheromones have been identified in <1% of formicine ant species (Regnier and Wilson, 1969; Ayre

and Blum, 1971; Kistner and Blum., 1971; Löfqvist, 1976; Duffield et al., 1977; Bradshaw et al., 1979; Hefetz and Orion, 1982; Hefetz and Lloyd, 1985; Kohl et al., 2001; Fujiwara-Tsujii et al., 2006; Witte et al., 2007; Lenz et al., 2013; Cerdá et al., 2014; Zhang et al., 2015). Alarm-recruitment pheromones of formicine ants typically comprise formic acid and/or aliphatic alkanes (Regnier and Wilson, 1969; Ayre and Blum, 1971; Kistner and Blum., 1971; Löfqvist, 1976; Duffield et al., 1977; Bradshaw et al., 1979; Hefetz and Orion, 1982; Hefetz and Lloyd, 1985; Kohl et al., 2001; Fujiwara-Tsujii et al., 2006; Witte et al., 2007; Lenz et al., 2013; Zhang et al., 2015). Substrate-borne vibratory signals, which are produced by workers slamming their body against substrate, have been characterized in only two species of *Camponotus* (Markl and Fuchs, 1972; Fuchs, 1976). Multi-modal communication in formicine ants is reported only as an unpublished observation in one species of *Camponotus* (Hölldobler, 1999).

Myrmica rubra is an aggressive soil-dwelling ant that is native to Eurasia but has invaded the east and west coasts of North America (Wetterer and Radchenko, 2010). First records of *M. rubra* show its arrival in the Northeastern US in the early 1900s (Groden et al., 2005). Introductions into western Canada and the northwestern United States (US) are likely from separate introductions from Eurasia (Naumann and Higgins, 2015). Colonies are polygynous and polydomous and – in their invasive range – reach high nest densities due to budding rather than mating flight dispersal behaviour (Groden et al., 2005; Naumann et al., 2017). The painful sting and high nest densities of *M. rubra* render areas unusable for humans, and *M. rubra* activities alter arthropod and plant communities (Naumann and Higgins, 2015; Meadley-Dunphy et al., 2020). *Myrmica rubra* workers tend aphids, are generalist scavengers and predators (Wetterer and Radchenko, 2010; Garnas et al., 2014), and they distinguish between sugar molecules (Boevé and Wäckers, 2003).

For taxonomic diversity, I included two additional ant taxa in my studies of ant foraging ecology and bait development but I did not study these species as extensively as *C. modoc* and *M rubra*. *Lasius niger* is a widespread (Janicki et al., 2016; Guénard et al., 2017), temperate, soil-dwelling ant that regularly tends aphids, and prefers aphid-derived sugars such as melezitose to common sugars such as sucrose (Völkl et al., 1999; Woodring et al., 2004). *Lasius niger* also preferentially feeds on diverse amino acid blends (Woodring et al., 2004; Madsen et al., 2017) but any potential preference for specific amino acids is not known. *Formica aserva* is a brood-raiding ant, nesting in

woody debris (Higgins et al., 2017) such as stumps (Wu and Wong, 1987). It tends aphids (Phillips and Willis, 2005) and collects insect prey (Savolainen and Deslippe, 2001) but nutrient preferences are not yet documented. *Formica oreas* builds conspicuous thatch mounts (Risch et al., 2008) and is present in western Canada and the US (Janicki et al., 2016; Guénard et al., 2017). Workers forage intensely on sugar at high concentrations (Crawford and Rissing, 1983)

1.5. Overview of thesis chapters

In my thesis, I investigated the communication signals that ants use during nest defense and foraging, and the cues they exploit to locate and discern between nutrient resources. My specific research objectives were to understand how ants communicate the location of resources and the presence of threats, respond to mono-modal signals (pheromone or vibration) or a bi-modal signal complex (pheromone and vibration), and how ants locate and select food resources. Lastly, I investigated whether the ants' nutrient preferences can be used to improve the appeal of food baits for control of pest ants.

My thesis consists of nine chapters. The first chapter (Chapter 1) provides a concise overview of my field of study. Chapters 2–9 are Research Chapters which represent new findings, with near-identical versions of these Chapters already published in peer-reviewed journals or currently in peer review. As Part I of my thesis, Chapters 2–3 focus on alarm communication signals of *C. modoc,* whereas Chapters 4–9, as Part II of my thesis, focus on ant foraging ecology, nutrient consumption, and bait development for ant control. In Chapters 4–9, I work mainly with *C. modoc* and *M. rubra* species, but also include *Formica oreas, F. aserva,* and *L. niger* for taxonomic breadth.

In Chapter 2, I study the alarm-recruitment pheromone components used by distressed *C. modoc* worker ants. My objectives were to: (1) ascertain the glandular sources of the alarm-recruitment pheromone components, (2) elucidate their molecular structures, and (3) determine whether these components are indeed discharged by alarmed ants. To this end, we extracted the content of the poison and Dufour's gland, and analysed extracts by coupled gas chromatography - mass spectrometry. In olfactometer bioassays, gland extracts were attractive to *C. modoc* workers. Testing the synthetic alarm pheromone blend revealed that formic acid, benzoic acid and 4 aliphatic

alkanes mediate the attraction of ants. The synthetic blend was also attractive to ants in still air arena bioassays. All components, except benzoic acid, were detected in sprays from distressed ants.

Chapter 3 builds on (*i*) the results of Chapter 2 that the alarm pheromone of *C. modoc* attracts nestmates, and (*ii*) literature reports that vibratory signals prompt carpenter ants to stand still (freeze) or run fast, thus helping predator evasion. For my studies in Chapter 3, I assume that nestmates being attacked by a vertebrate predator engage in both pheromonal and vibratory signalling, and I argue that orientating towards signallers under vertebrate predator attack is maladaptive and not beneficial to the colony. Within this framework, I then tested the hypotheses (1) that vibratory alarm signals cause freezing, rapid running but not attraction of *C. modoc* nestmates, and (2) that bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk. In Laser Doppler vibrometry recordings, I show that the ants' vibratory signals readily propagate through ant nest lamellae, and thus quickly inform nestmates of perceived threats. With a speaker modified to record and deliver vibratory signals, I obtained drumming signals of distressed ants on a Douglas fir veneer, and bioassayed signal effects on ants in an arena with a suspended veneer floor. In response to playback of vibratory signals, ants ran rapidly, or froze, but did not approach the vibratory signals. Exposed to alarm pheromone, ants frequently visited the pheromone source. However, concurrently exposed to both alarm pheromone and vibratory signals, ants visited the pheromone source less often but spent more time 'frozen'. I argue that the ants' modulated responses to bi-modal signals is adaptative but I admit that the reproductive fitness benefits are still to be quantified.

Chapter 4 is the first of several chapters that investigated foraging behaviour of ants. In Chapter 4, I studied how *C. modoc* worker ants locate new food resources. I posited that forager ants accrue significant energy savings, if they were able to sense and orient toward odor plumes of both carbohydrate and protein food sources. I further posited that if worker ants, like other flightless insects, had reduced olfactory acuity, they would not recognize the specific odor signatures of diverse carbohydrate and protein sources, but they may be able to orient toward those odorants that are shared between (macronutrient) food sources. Within this framework, I then tested the hypotheses that (1) food sources rich in carbohydrates (aphid honeydew, floral nectar) and rich in proteins (bird excreta, house mouse carrion, cow liver infested or not with fly maggots)

all prompt long-distance, anemotactic attraction of worker ants, and (2) attraction of ants to plant inflorescences (fireweed, *Chamaenerion angustifolium*; thimbleberry, *Rubus parviflorus*; and hardhack, *Spiraea douglasii*) is mediated by shared floral odorants. In moving-air Y-tube olfactometer bioassays, ants were attracted to two of four carbohydrate sources (thimbleberry and fireweed), and one of four protein sources (bird excreta). Headspace volatiles of fireweed, thimbleberry, and bird droppings were collected by dynamic headspace aerations and analyzed by gas chromatography-mass spectrometry. Synthetic odor blends of thimbleberry (7 components), fireweed (23 components), and bird excreta (38 components) were prepared and tested in Y-tube olfactometer bioassays. Ants were attracted to synthetic blends of thimbleberry and fireweed but not bird excreta, indicating that only the two floral blends contained all essential attractants. A blend of components shared between thimbleberry and fireweed was not attractive to ants. My data support the conclusion that *C. modoc* worker ants can sense and orient toward both carbohydrate and protein food sources. As ants were selective in their responses to carbohydrate and protein resources, it seems that they can discern between specific food odor profiles and that they have good, rather than poor, olfactory acuity.

In Chapter 5, I aimed to identify the trail pheromone that *C. modoc* worker ants deposit to guide nestmates to a food resource they have located. We excised and extracted the ants' hindgut – the typical source of trail pheromone in formicine ants – and we measured the distance ants walked in response to hindgut extract presented as a continuous trail. In gas chromatographic-electroantennographic detection and gas chromatographic-mass spectrometric analyses of hindgut extracts, we identified five candidate components: 2,4-dimethylhexanoic acid, 2,4-dimethyl-5-hexanolide, pentadecane, dodecanoic acid and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin. In a series of trail-following experiments, we determined that ants followed trails of synthetic 2,4-dimethyl-5-hexanolide, a blend of the five compounds, and hindgut extract over similar distances, indicating that the hexanolide accounted for the entire behavioral activity of the hindgut extract. Further analyses and bioassays with racemic and stereoselectively synthesized hexanolides revealed that the ants produce, and respond to, the (2*S*,4*R*,5*S*)-stereoisomer. The (2*S*,4*R*,5*S*)-stereoisomer also attracted *C. modoc* ants in Y-tube olfactometers over distance, revealing a previously unknown dual function. As the same stereoisomer is a trail pheromone component in several

Camponotus congeners, there is evidence for significant overlap in trail pheromone components among *Camponotus* congeners.

In Chapter 6, I shift from studying resource location by ants to resource selection by ants. I focused on carbohydrate resources, bearing in mind that ants select sustained carbohydrate resources, such as aphid honeydew, according to sugar type, volume, and concentration. I tested the hypotheses (H1–H3) that *C. modoc* seek honeydew excretions from *Cinara splendens* aphids based solely on the presence of sugar constituents (H1), prefer sugar solutions containing aphid-specific sugars (H2), and preferentially seek sugar solutions with higher sugar content (H3). I further tested the hypothesis (H4) that workers of both *C. modoc* and *M. rubra* selectively consume particular mono-, di- and trisaccharides. In choice bioassays with *C. modoc* colonies, sugar constituents in honeydew (but not aphid-specific sugar) as well as sugar concentration affected consumption. Both *C. modoc* and *M. rubra* foragers consumed more fructose compared to other monosaccharides (xylose, glucose) and sucrose to other disaccharides (maltose, melibiose, trehalose). Conversely, when we offered a choice between the aphid-specific trisaccharides raffinose and melezitose, *C. modoc* and *M. rubra* favoured raffinose and melezitose, respectively. Testing the favourite mono-, di- and trisaccharide head-to-head, both ant species favoured sucrose. I conclude that while both sugar type and sugar concentration are the ultimate cause for consumption by foraging ants, strong recruitment of nest-mates to superior sources is probably the major proximate cause.

In Chapter 7, I continue to study resource selection by ants but I shift focus from carbohydrate to amino acid resources. I drew on literature reports that foraging ants collect amino acids and proteins for developing larvae in their colony, and that both essential amino acids (EAAs; some considered toxic to ants) and non-essential amino acids (non-EAAs) are important building blocks of proteins. As EAAs cannot be synthesized by animals and must be obtained from their diet, I predicted that ants preferentially consume essential EEAs. Prior to my study, it was not well known whether ants specifically forage for EAAs, and how EAAs affect ant colony growth. Using *M. rubra* and *C. modoc* as model species, I tested the hypotheses that (1) *M. rubra* and *C. modoc* colonies with brood preferentially consume EAAs rather than non-EAAs; (2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth; and (3) *M. rubra* workers feeding on sucrose and EAAs

die sooner than workers feeding on sucrose and non-EAAs (which are considered less toxic). In laboratory choice experiments, we found that colonies of *M. rubra* and *C. modoc* preferentially consumed EAAs rather than non-EAAs. *M. rubra* colonies that were provided both EAAs and non-EAAs, produced more larvae, but not more workers and queens, than colonies provided only EAAs or non-EAAs. Measuring time-to-death in *M. rubra* workers that were removed from their colonies, workers that consumed sucrose and EAAs died sooner than workers that consumed sucrose and non-EAAs, possibly because they could not feed EAAs to larvae. My data support the conclusion that EAAs on their own, while critically important, are insufficient for ant colony growth.

In Chapter 8, I drew on results of Chapters 6 and 7 that sucrose and EAAs are key macro-nutrients for ants, realizing that the relative contribution of these macro-nutrients in momentary and season-long foraging responses by ants had hardly been investigated. Therefore, using *C. modoc* and *M. rubra* as model species, we (1) tested preferential consumption of various macro- and micro-nutrients, (2) compared consumption of preferred macro-nutrients, (3) investigated seasonal shifts in nutrient preferences, and (4) tested whether nutrient preferences of *C. modoc* and *M. rubra* pertain to *L. niger* and *F. aserva*. In laboratory and field experiments, we measured nutrient consumption by weighing Eppendorf tubes containing aqueous nutrient solutions before and after feeding by ants. Laboratory *C. modoc* colonies consumed nitrogenous urea and essential amino acids (EAAs), whereas *M. rubra* colonies consumed sucrose. Field colonies of *C. modoc* and *M. rubra* preferentially consumed EAAs and sucrose, respectively, with no sustained shift in preferred macro-nutrient over the course of the foraging season. The presence of a less preferred macro-nutrient in a nutrient blend did not diminish the blend's 'appeal' to foraging ants. Sucrose and EAAs singly and in combination were equally consumed by *L. niger,* whereas *F. aserva* preferred EAAs. As baits containing both sucrose and EAAs were consistently consumed by the ants studied in this Chapter, I propose that sucrose and EAAs should be considered as bait constituents for pest ant control.

Chapter 9 represents the 'applied sciences' project in my thesis, where I strive to put knowledge acquired in preceding chapters to work for pest ant control. Chapter 9 is based on my findings in Chapter 8 that an aqueous ant bait [sucrose (4.55%), essential amino acids (1%), water] is highly appealing to multiple ant species throughout the foraging season. My specific research objectives in this Chapter were to: (1) assess bait

lethality to diverse species of ants (*M. rubra, C. modoc*, *F. obscuripes*); (2) test the effect of boric acid dose on mortality of *M. rubra* workers and colonies; (3) compare consumption, and demise timeline, of lethal liquid baits and lethal gel baits; and (4) investigate whether lethal liquid baits reduce the size of *M. rubra* field colonies. In laboratory experiments, we found that the bait induced rapid mortality in all three species of ants tested. Increasing the dose of boric acid from 1% to 5.4% accelerated mortality of worker ants, but not queen ants, in *M. rubra* colonies, indicating that 1% boric acid is sufficiently lethal. Worker ants of *M. rubra* consumed more liquid baits to gel baits of identical nutrient composition, with liquid baits accelerating worker mortality. In a field experiment in a public park heavily infested with *M. rubra*, the 12 treatment colonies provided with a lethal liquid bait (4.55% sucrose; 1% EAA; 1% boric acid) worker counts over 114 days significantly declined, whereas the 12 control colonies provided with the corresponding non-lethal bait did not. I conclude that the bait, with appropriately adapted bait deployment protocol, should be tested for control of other pest ants, particularly those that preferentially feed on liquid foods.

1.6. References

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Chapter 2.

A blend of formic acid, benzoic acid, and aliphatic alkanes mediates alarm recruitment responses in western carpenter ants, *Camponotus modoc*

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2.1. Abstract

Formicine ants in distress spray alarm pheromone which typically recruits nestmates for help. Studying the western carpenter ant, *Camponotus modoc* Wheeler (Hymenoptera: Formicidae), our objectives were to (1) determine the exocrine glands that contain alarm recruitment pheromone, (2) identify the key alarm recruitment pheromone components, and (3) ascertain the pheromone com- ponents that are discharged by distressed ants. In Y-tube olfactometer experiments, extracts of poison glands, but not of Dufour's glands, elicited anemotactic responses from worker ants. Gas chromatographic-mass spectrometric analyses of poison gland extracts revealed the presence of (1) aliphatic alkanes (undecane, tridecane, pentadecane, heptadecane), (2) aliphatic alkenes [(*Z*)-7-pentadecene, (*Z*)-7- and (*Z*)-8-heptadecene], (3) two acids (formic, benzoic), and (4) other oxygenated com- pounds (hexadecan-1-ol, hexadecyl formate, hexadecyl acetate). Testing the responses of worker ants in Y-tube olfactometers to complete and partial synthetic blends of these compounds revealed that the acids and the alkanes are essential alarm pheromone components. In two-choice arena bioassays, micro-locations treated with synthetic alarm pheromone recruited worker ants. Acids and alkanes were abundant in the poison gland and the Dufour's gland, respectively, suggesting that the alarm pheromone components originate from both glands. Moreover, alarm pheromone sprays of ants differed in that all sprays contained formic acid but only some also contained alkanes, implying that ants can independently discharge the content of either one or both glands in accordance with the type of distress incident they experience.

2.2. Introduction

The nests of social insects, including ants, contain many developing larvae and pupae, brood-tending adults, and food stores, and thus are a nutrient-rich source for prospective predators and parasites (Ayasse & Paxton, 2002). Constructed ant nests represent investments made by entire colonies, housing their offspring as well as their queen(s), which represent(s) their entire reproductive potential. Consequently, nest defense is of paramount importance (Seeley, 1985; Ayasse & Paxton, 2002). To reduce the adverse impact of nest incursions, it is adaptive for ants to respond collectively to threats (Blum, 1985). In turn, a collective response to any threat is contingent upon a rapid and effective communication system (Verheggen et al., 2010).

Pheromones are commonly used by ants to coordinate group tasks including nest defense (Hölldobler & Wilson, 1990). When threatened, ants may spray alarm pheromones and discharge defense chemicals which both sub- due a predator or parasite and elicit distinct responses from nestmates such as attraction and aggression (Hölldobler & Wilson, 1990). Alarm pheromones originate from diverse exocrine glands (e.g., mandibular, poison, and Dufour's), and often comprise multiple components, some of which are of low molecular weight that facilitate rapid information conveyance (Hölldobler & Wilson, 1990; Morgan, 2008).

Alarm pheromones and corresponding behavioral responses have been studied in seven genera (*Formica, Cataglyphis, Camponotus, Lasius, Nylanderia, Oecophylla*, and *Paratrechina*) of formicine ants (Regnier & Wilson, 1969; Ayre & Blum, 1971; Kistner & Blum, 1971; Löfqvist, 1976; Duffield et al., 1977; Bradshaw et al., 1979; Hefetz & Orion, 1982; Fujiwara-Tsujii et al., 2006; Witte et al., 2007; Lenz et al., 2013; Zhang et al., 2015). Most of these studies tested responses of ants to excised exocrine glands or syn- thetic gland constituents. Pheromone blends actually discharged by ants *in situ* were analyzed in only a single study with the carpenter ant *Camponotus obscuripes* Mayr (Fuji-wara-Tsujii et al., 2006).

Carpenter ants, belonging to the genus *Camponotus*, excavate tunnel galleries in wood to create nesting sites (Hansen & Klotz, 2005). With >1000 species, they are one of the most species-rich ant genera, inhabiting nearly all terrestrial ecosystems (Bolton, 1995; Janicki et al., 2016; Guénard et al., 2017). Alarm pheromones of *Camponotus*

originate from poison and Dufour's glands (Ayre & Blum, 1971; Hefetz & Orion, 1982; Kohl et al., 2001; Fujiwara- Tsujii et al., 2006). Distressed ants of *C. obscuripes* discharge formic acid from their poison gland and aliphatic hydrocarbons from their Dufour's gland (Fujiwara-Tsujii et al., 2006). Ants avoided formic acid but oriented toward extracts of the Dufour's gland, with decane and undecane as the primary attractants (Fujiwara-Tsujii et al., 2006). Formic acid attracts worker ants of *Camponotus gestroi* Emery, *Camponotus sericeus* Fabricius, *Camponotus thoracicus fellah* Emery, *Camponotus balzani* Emery, *Camponotus castaneus* Latreille, *Camponotus sericeiventris* Guérin-Méneville, and *Camponotus socius* Roger (Hefetz &Orion, 1982; Kohl et al., 2001, 2003), whereas undecane elicits running, attraction, and aggression in workers of *C. thoracicus fellah, C. gestroi*, and *C. socius* (Hefetz & Orion, 1982; Kohl et al., 2001). Formic acid and undecane in combination are more effective than on their own in prompting attraction and settling responses by worker ants of *Camponotus pennsylvanicus* De Geer, *Camponotus herculeanus* L., and *Camponotus americanus* Mayr (Ayre & Blum, 1971).

The western carpenter ant, *Camponotus modoc* Wheeler (Hymenoptera: Formicidae), is common in forested and urban areas along the west coast of Canada and the USA (Hansen & Akre, 1985). Ants excavate nesting sites in tree logs, stumps, and human-made wooden structures. Nests are monogynous, with up to 50 000 workers occupying several nesting sites (Hansen & Akre, 1985; Akre et al., 1994). In a previous study, extracts of the poison gland and the Dufour's gland were not attractive to *C. modoc* workers (Hansen & Akre, 1985), but the bioassay response criterion – number of worker ants occupying the site of extract application – may have discounted the rapid movement of ants, and thus may not have fully grasped the behavioral effect of these extracts. Our objectives were to (1) determine the exocrine glands that contain alarm recruitment pheromone components, (2) identify the key alarm recruitment pheromone components, and (3) ascertain the pheromone components that are actually discharged by distressed ants.

2.3. Materials and methods

2.3.1. Ant rearing

Acquisition and maintenance of ant nests have previously been described in detail (Renyard et al., 2019). Briefly, *C. modoc* nests were excised from forest logs and maintained in an outdoor undercover area of the Science Research Annex of Simon Fraser University, where ants experience natural cycles of light and temperature throughout the year. We housed ant-infested logs in large plastic bins connected via Nalgene tubing to glass aquaria which served as the ants' foraging area provisioned with insect prey, honey, apples, canned chicken, and 20% sugar water ad libitum. We used worker ants from four nests in experiments.

2.3.2. Preparation of poison and Dufour's gland extracts (2016)

We collected worker ants into a glass holding tube (1.8 × 25 cm) and cold-euthanized them in a -15 °C freezer. Removing one ant at a time from the freezer, we used 20 ants to excise and extract their poison gland (with reservoir) and Dufour's gland in two separate pooled samples. We dissected ants in distilled water under a dissection microscope [Zeiss, Oberkochen (formerly Jena), Germany], using dissection scissors and fine forceps (Fine Science Tools, North Vancouver, BC, Canada), and placed excised glands into 4-ml glass vials (VWR, Radnor, PA, USA) which contained dichloromethane (DCM; 0.5 ml) (EMD Millipore, Billerica, MA, USA) and resided on ice for the duration of the dissections. We macerated the glands with a glass rod and left them to extract at room temperature for 10 min. Prior to analyses, we filtered extracts through a glass pipette stuffed with glass wool into a clean 4-ml vial capped with a Teflon-lined lid. Between dissections of individual ants, we cleaned tools with DCM. We used these extracts for behavioral experiments 1 and 2, and for chemical analyses (2016) of the poison gland and reservoir.

2.3.3. Analyses of poison and Dufour's gland extracts by coupled gas chromatography-mass spectrometry

We analyzed aliquots of gland extracts on a Varian Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 column (50 m × 0.25

mm i.d.; Agilent Technologies, Santa Clara, CA, USA), using helium as the carrier gas (35 cm s^{-1}). The oven temperature program was as follows: 50 °C (held for 5 min), 10 °C per min to 280 °C (held for 10 min). The injector port, ion trap, and transfer line were set to 250, 200, and 280 °C, respectively. We identified compounds in extracts by comparing their retention indices (Van den Dool & Kratz, 1963) and mass spectra with those of authentic standards. We determined the double bond positions of unsaturated hydrocarbons by treating 50-µl aliquots of extracts with dimethyl disulfide (DMDS) (Dunkelblum et al., 1985). We separated *E*-and *Z*-isomers of unsaturated hydrocarbons using the same GC program as above except that the final temperature was 130 °C which was held for 30 min. To determine the presence and amount of formic acid which chromatographs poorly, we derivatized the acid to decyl formate (Neises & Steglich, 1978) which chromatographs well. To this end, we treated 25 µl of each extract with dimethylaminopyridine (12.5 µg), dicyclohexylcarbodiimide (27.5 µg), and decan-1-ol in large excess (125 µg), keeping the reaction mixture at room temperature for 24 h.

2.3.4. Purchase and syntheses of candidate alarm pheromone components

(*Z*)-7-Pentadecene, (*Z*)-8-heptadecene, and (*Z*)-7-heptadecene were synthesized in our laboratory. Undecane, tridecane, pentadecane, heptadecane, hexadecan-1-ol, hexadecyl formate, hexadecyl acetate, and benzoic acid were all purchased from Sigma-Aldrich (St Louis, MO, USA) with 99% purity. Formic acid was purchased from Anachemia Science (Rouses Point, NY, USA).

2.3.5. Minimizing visual bias in both Y-tube olfactometer and arena bioassays

To minimize any potential visual bias, we ran both Y-tube olfactometer and arena bioassays under a metal scaffold (123 × 57 × 36 cm) enclosed in black fabric, with a small gap in the fabric to allow recordings of ant behavior. We illuminated the bioassay assemble from above with two 32-W fluorescent lights (121.9 cm; Philips, Amsterdam, The Netherlands).

2.3.6. General design of Y-tube olfactometer bioassays

We tested distance attraction of worker ants to test stimuli in Pyrex glass Y-tube olfactometers (2.5 cm inner diameter, 22.5-cm-long main stem, 19-cm-long side arms, 120° angle of arms; Derstine et al., 2017), using an experimental design and protocol previously described in detail (Renyard et al., 2019).

To initiate a bioassay, we detached the Nalgene tubing (see above) that connects a nesting bin to a foraging area and plugged the tubing with a cotton ball. For each replicate, we isolated a single ant from a randomly selected colony. To do this, we removed the cotton ball from the tubing and inserted the tubing into a glass holding tube (25 cm long, 1.8 cm inner diameter), thus enabling an outgoing ant (presumably a forager) to exit the Nalgene tubing and to enter the glass holding tube. We attached the holding tube to a vacuum pump drawing air at 0.5 l per min. We then prepared an olfactometer by placing a piece of cotton roll near the opening of each side arm, and by treating the roll with either the test stimulus (poison gland extract, Dufour's gland extract, or blend of synthetic candidate pheromone components) or a DCM control. We assayed all test stimuli at 0.5 ant equivalents (AEs) dissolved in DCM (12–15 μl) and used the corresponding amount of DCM (12–15 μl) for all control stimuli. We attached the Y-tube stem to the holding tube via a ground glass joint thus drawing (candidate) pheromone components toward an ant entering the Y-tube. We recorded the test stimulus which an ant approached first within 6 cm and continued to run bioassays until at least 20 singly tested ants had made a choice (except for experiments 12– 14, see below). We considered all ants that made no choice within 10 min non-responders and excluded them from statistical analyses. We used a clean Y-tube and holding tube for each replicate.

2.3.7. Specific Y-tube experiments

Attraction to poison and Dufour's gland extracts (experiments 1, 2; 2016).

We offered single worker ants choices between (1) poison gland extract of workers vs. a solvent control (experiment 1, n = 20) and (2) Dufour's gland extract of workers vs. a solvent control (experiment 2, n = 20) (Table 2.1).

Attraction to poison gland extract and to a synthetic blend (SB) of candidate alarm pheromone components (experiments 3, 4; 2017).

As poison gland extract, but not Dufour's gland extract, attracted worker ants in experiments 1 and 2 in 2016 (see Results), we prepared a fresh extract of poison glands (n = 36 in a pooled sample) in 2017, analyzed this sample (see column 2 in Table 2.2), and in parallel experiments 3 and 4 (which were run concurrently over several days) offered ants choices between (1) poison gland extract vs. a solvent control (experiment 3, n = 20), and (2) a synthetic blend (SB) of candidate alarm pheromone components vs. a solvent control (experiment 4, n = 20) (Table 2.1).

Bioassays to determine essential components in the alarm pheromone SB (experiments 5–8; 2017).

As the SB of alarm pheromone components was as attractive to worker ants as poison gland extract and thus contained all essential pheromone components (see Results), we aimed to determine these essential components in the SB. In parallel experiments 5–8, we tested the complete SB comprising hydrocarbons [undecane, tridecane, pentadecane, heptadecane, (*Z*)-7-pentadecene, (*Z*)-8- heptadecene, (*Z*)-7-heptadecene], oxygenated compounds (OCs; hexadecan-1-ol, hexadecyl formate, hexadecyl acetate), and acids (benzoic, formic) (experiment 5, n = 20), and partial SBs lacking hydrocarbons (experiment 6, n = 20), OCs (experiment 7, n = 20), or acids (experiment 8, n = 20), each vs. a solvent control (Table 2.1).

Bioassays to determine the essential acid(s) in the alarm pheromone SB (experiments 9–11; 2017).

As the attractiveness of SB was contingent upon the presence of acids but not OCs (see Results), we aimed to determine the essential acid(s) in a partial SB (SB minus OCs). In parallel experiments 9-11, we tested SB minus OCs (positive control; experiment 9, n = 20), SB minus OCs lacking benzoic acid (experiment 10, n = 20), and SB minus OCs lacking formic acid (experiment 11, n = 20), each blend vs. a solvent control (Table 2.1).

Bioassays to determine the essential hydrocarbons in the alarm pheromone SB (experiments 12–14; 2017).

As the attractiveness of SB was also contingent upon the presence of hydrocarbons (see Results), we aimed to determine the essential hydrocarbons in the

blend. In parallel experiments 12–14, we tested SB minus OCs (positive control; experiment 12, n = 26), SB minus OCs lacking alkenes (experiment 13, n = 25), and SB minus OCs lacking alkanes (experiment 14, n = 18), each blend vs. a solvent control (Table 2.1). In experiment 14, the treatment stimulus was hardly attractive to ants (see Results), prompting many non-responders.

Behavioral responses in arena bioassays to alarm recruitment pheromone-treated micro-locations (experiment 15; 2017)

As ants are likely to emit and sense the alarm recruitment pheromone in the presence of nestmates, and in a spacious setting, we tested whether groups of five worker ants entering a large foraging arena discern micro-locations treated with alarm pheromone. We placed two circular Whatman filter papers (90 mm diameter; Sigma-Aldrich) 41 cm apart from one another inside a plexiglass bioassay arena (64 × 44 × 10 cm; Figure 2.1), and by random assignment treated one filter paper with the complete synthetic blend of candidate pheromone components (0.5 ant equivalents) and the other filter paper with a solvent control (Table 2.1; n = 20). To commence a bioassay, we placed a 15-ml Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) containing five worker ants from a randomly selected colony into the bioassay arena such that the tube's tapered tip laid flush with the arena floor and was equidistant to each of two test stimuli. We then removed a cotton plug from a 0.7-cm-diameter hole cut in the tube's tip, allowing the ants to calmly exit the tube and walk into the arena. Once the first ant had entered the arena, we video recorded (Canon FS300 video camera; Canon, Tokyo, Japan) the ants' behavior for 150 s. We later reviewed the footage in slow motion with VLC Media Player v. 2.2.6 and recorded the number of ant visits to test stimuli and the time ants spent on each filter paper. After each replicate, we wiped the arena and countertops with 70% ethanol and hexane and washed the Falcon tube with water and soap (Sparkleen; Thermo Fisher Scientific).

2.3.8. Acquisition and analysis of ant alarm sprays (experiment 16; 2018)

To analyze the composition of alarm pheromone sprayed by distressed ants, we allowed a single worker ant (n = 12) in the foraging area to walk into a 3-ml vial. We then disturbed this ant with a pair of soft forceps (BioQuip, Rancho Dominguez, CA, USA). As

soon as she released a liquid spray, we removed her, and immediately rinsed the vial with DCM (500 µl), analyzing aliquots of the rinse by GC-MS.

2.3.9. Analyses of chemical components in extracts of the poison gland, poison gland reservoir, and Dufour's gland (2017)

An alarm pheromone spray may comprise chemical com- ponents originating from the poison gland and the Dufour's gland. The poison gland of *C. modoc* consists of a thin disc of compact fine tubules (the exocrine gland) which resides as a cap on top of the poison gland reservoir, whereas the bilobed Dufour's gland is situated near the base of the poison gland reservoir (Hansen & Akre, 1985). To track the origin of pheromone components, we excised 22 poison glands, 20 poison gland reservoirs, and 19 Dufour's glands, and extracted them as three separate pooled samples (see columns 3–5 in Table 2.2) in 4-ml glass vials, each containing 500 µl of DCM and residing on ice during dissections. We macerated tissue with a glass rod and allowed 10 min for compound extraction to proceed at room temperature. We analyzed 2-µl aliquots of each extract by GC-MS.

Statistical analysis We analyzed data and produced graphics in R v.3.2.2 and RStudio v.1.0.136 (R Core Team, 2018). In Y-tube olfactometer experiments 1–14, we used v2 tests to compare first-choice response data of ants to various test stimuli each vs. a solvent control stimulus. For arena experiment 15, we compared the mean number of ant visits at two micro-locations, and the time ants spent at these locations, with a paired t-test.

2.4. Results

2.4.1. Results Attraction to poison and Dufour's gland extracts (experiments 1, 2)

In Y-tube olfactometers, worker ants were attracted to poison gland extracts of workers (χ^2 = 12.8, P = 0.0003) but not to Dufour's gland extracts of workers (χ^2 = 0.2, P = 0.65, both d.f. = 1; Figure 2.2) each tested against a solvent control stimulus.

2.4.2. Identification of candidate pheromone components in poison gland extracts

As poison gland extracts, but not Dufour's gland extracts, attracted worker ants, we prepared and analyzed a fresh poison gland extract in 2017, and identified the following components: undecane (mean amount per ant in pooled sample: 20 ng), tridecane (10 ng), (*Z*)-7-pentadecene (1 ng), pentadecane (3 ng), (*Z*)-7- and (*Z*)-8-heptadecene (1 ng each), heptadecane (1 ng), hexadecan-1-ol (20 ng), hexadecyl acetate (2 ng), hexadecyl formate (30 ng), and formic acid (3474 ng) (Table 2.2). Benzoic acid was not detected in the 2017 extracts, but it was above detection threshold in 2016 poison gland extracts, being present at 0.25% relative to formic acid. Bearing in mind that important pheromone components may be present in gland extracts in only trace amounts (Gries et al., 2002) and may not always be detectable by GC-MS, we took a comprehensive approach and included benzoic acid in the preparation of samples for bioassays. The DMDS treatment revealed an ion pair (m/z 145/159) diagnostic for (*Z*)-7-pentadecene, and equally abundant ion pairs (m/z 145/187, 159/173) diagnostic for (*Z*)-7- and (*Z*)-8-heptadecene being present in near equal amounts. The cis-geometry of all alkenes could be determined because trans-isomers (2-3% in synthetic standards) eluted distinctively later than cis-isomers when chromatographed using an appropriate GC temperature program (see above).

2.4.3. Attraction to poison gland extract and to a synthetic blend (SB) of candidate alarm pheromone components (experiments 3, 4)

In parallel experiments 3 and 4, worker ants were attracted to poison gland extracts of workers (χ^2 = 7.2, P = 0.007) and to a synthetic blend of candidate alarm recruitment pheromone components identified in poison gland extracts (χ^2 = 16.2, P<0.0001, both d.f. = 1; Figure 2.3).

2.4.4. Bioassays to determine essential components in the alarm recruitment pheromone SB (experiments 5–8)

Attraction of worker ants was dependent upon the chemical composition of the SB. Ants were attracted to the complete SB (experiment 5; χ^2 = 7.2, P = 0.007) and the SB minus OCs (experiment 7; χ^2 = 9.8, P = 0.002) but not to the SB lacking

hydrocarbons (experiment 6; $\chi^2 = 0.2$, P = 0.65) or acids (experiment 8; $\chi^2 = 0.8$, P = 0.37, all d.f. = 1; Figure 2.4).

2.4.5. Bioassays to determine the essential acid(s) in the alarm recruitment pheromone SB (experiments 9–11)

Ants were attracted to the SB minus OCs (experiment 9; $\chi^2 = 5$, P = 0.025) but not to the SB minus OCs lacking either benzoic acid (experiment 10; $\chi^2 = 0.2$, P = 0.65) or formic acid (experiment 11; $\chi^2 = 0.8$, P = 0.37, all d.f. = 1; Figure 2.4).

2.4.6. Bioassays to determine the essential hydrocarbons in the alarm recruitment pheromone SB (experiments 12–14)

Ants were attracted to the SB minus OCs (experiment 12; $\chi^2 = 3.85$, P = 0.05), the SB minus OCs lacking alkenes (experiment 13; $\chi^2 = 6.76$, P = 0.009) but not to the SB minus OCs lacking alkanes (experiment 14; $\chi^2 = 0.89$, P = 0.3, all d.f. = 1; Figure 2.4).

2.4.7. Behavioral responses of ants to alarm recruitment pheromone-treated micro-locations (experiment 15)

In two-choice arena bioassays, ants visited more often the micro-location treated with synthetic alarm recruitment pheromone than the micro-location treated with a solvent control (t = -4.021, d.f. = 19, P = 0.0007; Figure 2.5A). Similarly, ants spent more time in the pheromone-treated micro-location than in the solvent control-treated micro-location (t = -4.611, d.f. = 19, P = 0.0002; Figure 2.5B).

2.4.8. Acquisition and analysis of ant alarm recruitment sprays (experiment 16)

Invariably, worker ants sprayed formic acid, but not benzoic acid, in detectable amounts (Table 2.3). Five out of 12 sprays revealed substantial amounts of the pheromonal alkanes, with two additional samples revealing trace amounts of alkanes.

2.4.9. Analyses of chemical components in extracts of the poison gland, poison gland reservoir, and the Dufour's gland

Pheromonal alkanes were most abundant in the Dufour's gland, present in intermediate amounts in the poison gland reservoir, and least abundant in the poison gland (Table 2.2, columns 3–5). Conversely, formic acid was most abundant in the poison gland and poison gland reservoir, with lower amounts present in the Dufour's gland. Benzoic acid was not detected in any of the three extracts.

2.5. Discussion

The alarm recruitment pheromone blend of *C. modoc* worker ants is complex, comprising two acids (formic and benzoic) and at least one of four aliphatic alkanes (undecane, tridecane, pentadecane, and heptadecane). A synthetic blend of these components elicits distance attraction of worker ants in Y-tube olfactometer assays and attracts ants to pheromone-treated micro-locations in arena assays. The blend, therefore, has the typical trade- marks of an alarming message that recruits nest mates to an incident of distress where help and concerted action of nest mates are required. The acid and hydrocarbon pheromone components of the blend appear to originate from both the poison gland and the Dufour's gland and are present not only in gland extracts but also in defense sprays of ants. Below, we shall elaborate on these conclusions.

Bioassaying the attractiveness of poison and Dufour's gland extracts in experiments 1 and 2 revealed that only the poison gland extract attracted worker ants. The next year, we therefore prepared a fresh poison gland extract, identified all chemical constituents, and prepared a syn- thetic blend of candidate alarm pheromone components. This synthetic blend, when tested at 0.5 ant equivalents in parallel with poison gland extract (experiments 3 and 4), was attractive to ants, indicating that it contained the essential alarm recruitment pheromone components. To determine these components, we tested the complete synthetic blend and blends lacking groups of organic molecules, such as acids and hydrocarbons, or lacking individual components. These experiments revealed that both acids and hydrocarbons are essential blend components (experiments 5, 6, and 8). Follow-up experiments further revealed that both formic acid and benzoic acid are essential alarm recruitment pheromone components

(experiments 9–11), and that the alkanes rather than the alkenes contribute to the attractiveness of the alarm recruitment pheromone blend (experiments 12– 14).

Poison gland extracts of *Camponotus* congeners contain similar sets of chemical constituents (Brophy et al., 1973; Kohl et al., 2001; Fujiwara-Tsujii et al., 2006). Poison gland extracts of *C. socius*, e.g., contain formic and benzoic acids as well as undecane and heptadecane (Kohl et al., 2001). Moreover, formic acid and aliphatic alkanes are consistently found in poison and Dufour's gland extracts, respectively, of *Camponotus spp.* (Ayre & Blum, 1971; Brophy et al., 1973; Hefetz & Orion, 1982; Ali et al., 1988; Haak et al., 1996; Kohl et al., 2001, 2003; Fujiwara-Tsujii et al., 2006). Formic acid is reported as a recruitment signal to trails in several *Camponotus* species (Traniello, 1977; Kohl et al., 2001, 2003). Dissimilar to our study, workers of *C. obscuripes* in two-way olfactometer assays avoided formic acid but were attracted to the aliphatic alkanes decane and undecane (Fujiwara-Tsujii et al., 2006). Benzoic acid, until this study, was not known to be an alarm recruitment pheromone component of *Camponotus*, but it has been reported as a constituent in pheromone blends of various insect taxa including the carpenter bee *Xylocopa hirsutissima* Maidl (Nishida et al., 1996) and the giant danaine butterfly *Idea leuconoe* Erichson (Gerling et al., 1989). That we detected benzoic acid in 2016 but not in 2017 gland extracts is likely due to an overall lower quantity of all pheromone components (except formic acid) in 2017 extracts. This decrease in pheromone titer (for which we have no definitive explanation) coupled with poor chromatography of acids are contributing factors that kept benzoic acid below detection threshold in 2017 extracts.

The ability of the alarm recruitment pheromone blend to recruit nest mates was also apparent in two-choice (arena) bioassays. The micro-location treated with the synthetic pheromone blend prompted both more and longer ant visits than the corresponding control location. In *C. obscuripes*, workers visited point locations treated either with Dufour's gland extract or its constituents decane and undecane more often than solvent controls (Fujiwara-Tsujii et al., 2006). Similarly, worker ants of *C. pennsylvanicus, C. herculeanus*, and *C. americanus* were attracted to paper cards treated with formic acid, undecane, or both (Ayre & Blum, 1971). Worker ants of these congeners settled on treated cards for more than 1 h contrasting with the rather brief visits of *C. modoc* worker ants to pheromone-treated filter paper in our study. These differential responses by ants can be attributed to divergent amounts of stimuli tested in

these studies [12 200 vs. 5 μg of formic acid (Ayre & Blum, 1971; this study)]. The fleeting attractive effect (2.5 min) of pheromone applications observed in arena assays could explain why Hansen & Akre (1985) did not find a behavioral effect associated with poison gland extract. When they recorded the ants' responses for the first time, 5 min after deployment of poison gland extract, the alarm recruitment effect may already have worn off, given the volatility particularly of the essential acid pheromone components.

The components of the *C. modoc* alarm recruitment pheromone blend seem to originate from both the poison and the Dufour's gland. This tentative conclusion is based on two considerations: (1) poison and Dufour's glands have dissimilar constituents or ratios of constituents – formic acid, in particular, was prevalent in poison gland extracts, whereas alkanes were prevalent in Dufour's gland extracts; and (2) the alarm pheromone sprays of ants differed in that all sprays contained formic acid but only some also contained alkanes, suggesting that ants can independently discharge the content of either one or both glands. This line of reasoning is further supported by reports that the poison gland and the Dufour's gland of formicine ants empty via separate ducts into the terminal gastral orifice (acidopore) (Hefetz & Orion, 1982). The variable propensity of ants to discharge alkanes could have been age- or caste-dependent (intrinsic factors) or may have been contingent upon the severity of the harassment as perceived by ants before discharging their spray. The relatively small amounts of the alkanes in poison gland extracts and of formic acid in Dufour's gland extracts may simply have originated as contaminants because we extracted each poison gland with its entire reservoir up to the acidopore, which discharges content from both the poison gland and the Dufour's gland.

In conclusion, we have identified the alarm recruitment pheromone blend of *C. modoc* worker ants. The alarm recruitment pheromone is sprayed by worker ants in distress and appears to represent a call for help. The pheromone likely also instigates nest defense, but this potential function will have to be experimentally tested in future studies.

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2.8. Tables

Table 2.1. Objectives (O) and stimuli tested for behavioral responses of *Camponotus modoc* ants

Exp	Test stimuli[1] (T)	No. single ants tested[4], or groups of ants (exp. 15)
O_1: Assess anemotactic attraction of ants to poison and Dufour's gland extracts		
1	T_1: poison gland extract; T_2: solvent control	28 (8)
2	T_1: dufour's gland extract; T_2: solvent control	20 (0)
O_2: Identify candidate alarm pheromone components in the poison gland		
O_3: Test anemotactic responses to synthetic blends (SB) of candidate alarm pheromone components		
3	T_1: poison gland extract; T_2: solvent control	24 (4)
4	T_1: SB[2]; T_2: solvent control	21 (1)
O_4: Determine the essential alarm pheromone components		
5	T_1: SB[2]; T_2: solvent control	24 (4)
6	T_1: SB minus hydrocarbons[2]; T_2: solvent control	24 (4)
7	T_1: SB minus OCs[2,3]; T_2: solvent control	23 (3)
8	T_1: SB minus acids[2]; T_2: solvent control	22 (2)
O_5: Determine the essential acid components		
9	T_1: SB minus OCs[2,3]; T_2: solvent control	20 (0)
10	T_1: SB minus OCs minus benzoic acid[2,3]; T_2: solvent control	20 (0)
11	T_1: SB minus OCs minus formic acid[2,3]; T_2: solvent control	21 (1)
O_6: Determine the essential hydrocarbon components		
12	T_1: SB minus OCs[2,3]; T_2: solvent control	26 (0)

13	T_1: SB minus OCs minus alkenes[2,3]; T_2: solvent control	27 (2)
14	T_1: SB minus OCs minus alkanes[2,3]; T_2: solvent control	27 (9)
O_7: Test ant visitation and time spent on pheromone treated micro-locations		
15	T_1: SB[2]; T_2: solvent control	20 groups of 5 ants each
O_8: Determine whether alarm pheromone components are discharged by distressed ants		
16	T_1: physically disturbing ants	12
O_9: Track glandular origin of pheromone components		

[1]Poison and Dufour's gland extracts and SB were all tested at 0.5 ant equivalents.

[2]SB: undecane (10 ng), tridecane (5 ng), pentadecane (1.5 ng), heptadecane (0.5 ng), (Z)-7-pentadecene (0.5 ng), (Z)-8-heptadecene (0.5 ng), (Z)-7-heptadecene (0.5 ng), hexadecan-1-ol (10 ng), hexadecyl formate (15 ng), hexadecyl acetate (1 ng), benzoic acid (6 ng), formic acid (5 µg).

[3]Oxygenated compounds (OCs): hexadecan-1-ol, hexadecyl formate, hexadecyl acetate.

[4]Numbers in parentheses are non-responding singly-tested ants

Table 2.2. Quantity (ng per ant) of chemical constituents present in extracts of the poison gland, the Dufour's gland, and separately the reservoir and gland of poison glands, of western carpenter ants, *Camponotus modoc*

Compounds	Poison gland + reservoir (n = 36)	Dufour's gland (n = 19)	Poison gland reservoir (n = 20)	Poison gland (n = 22)
Undecane	20	7016	278	17
Tridecane	10	3008	168	7
(*Z*)-7-Pentadecene	1	166	8	0.6
Pentadecane	3	629	37	1
(*Z*)-7 + (*Z*)-8-Heptadecene	1 + 1	102.5 + 102.5	5 + 5	0.7 + 0.7
Heptadecane	1	43	3	0.6
Hexadecan-1-ol	20	4	2	0
Hexadecyl acetate	2	48	7	129
Hexadecyl formate	30	369	27	6
Benzoic acid[1]	-	-	-	-
Formic acid[2]	3474	17	5133	3339

Data are based on dissections in 2017 of a group of 36 ants (column 2) and a group of 22 ants (columns 3-5), each column reporting data from a separate pooled sample; pooled (instead of single-ant) samples were collected, and analyzed in concentrated form, to enhance the probability of detecting trace components. In respectively 3 of 22 and 2 of 22 dissected ants, the Dufour's gland (column 3) or the poison gland reservoir (column 4) could not be successfully excised. Note: the variability in the amounts of chemical constituents between sources is likely due to both varied amounts present in sources at time of dissection, and variability in extractions of sources between dissections.

[1]The benzoic acid derivative remained below detection threshold of the mass spectrometer in this particular data set.

[2]Amounts are reported in ng of the ester derivative

Table 2.3. Chemical composition of discharges sprayed by distressed workers of the western carpenter ant, Camponotus *modoc*, taken from four nests. Ants sprayed into a 3-ml vial which was then rinsed with dichloromethane for analysis by gas chromatography-mass spectrometry

Nest	Ant	Amounts (ng) sprayed per ant				
		Formic acid[1,2]	Undecane	Tridecane	Pentadecane	Heptadecane
1	1	2901	430	30	32	4
	2	2316	1380	211	159	24
	3	1936	-	-	-	-
2	4	85	1518	1119	288	21
	5	215	-	3	1	0.2
	6	365	3148	1739	361	28
3	7	830	-	-	-	-
	8	732	883	867	431	28
	9	418	-	2	-	0.2
4	10	456	-	-	-	-
	11	653	-	-	-	-
	12	619	-	-	-	-

[1]Amounts are reported in ng of the ester derivative.

[2]The benzoic acid derivative remained below detection threshold of the mass spectrometer in this particular data set.

2.9. Figures

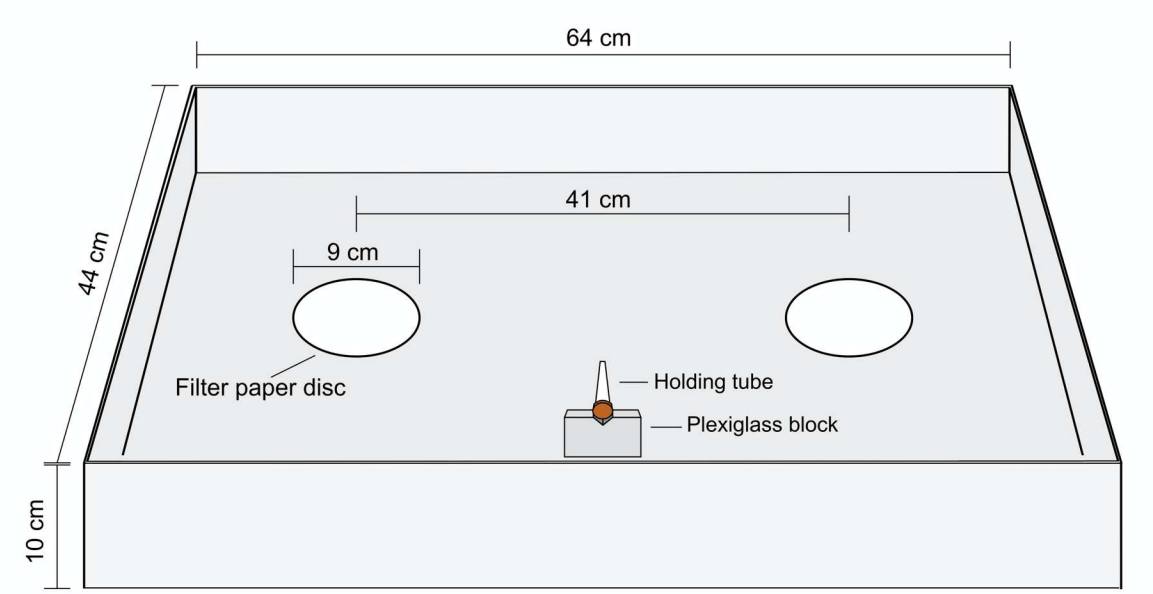

Figure 2.1. **Graphical illustration of the two-choice bioassay arena. For each replicate, five ants could exit the holding tube and respond to paper disc micro-locations treated with either synthetic alarm recruitment pheromone or a solvent control. The number of ant visits to these micro-locations and the time they spent there were recorded as response criteria.**

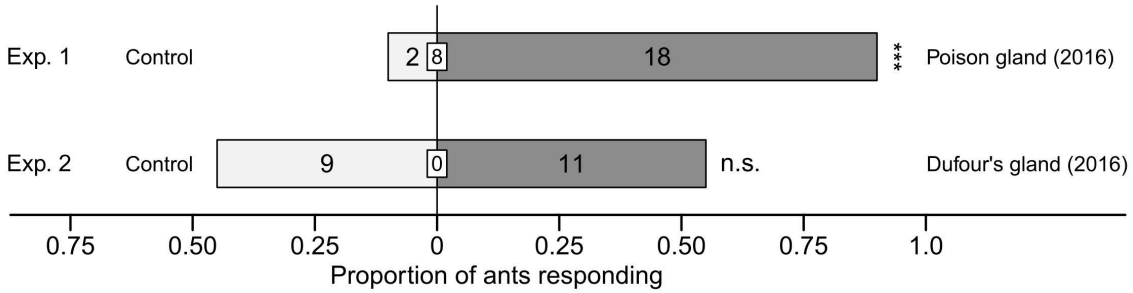

Figure 2.2. **Proportion of *Camponotus modoc* worker ants responding in two-choice Y-tube olfactometers to poison gland extract of workers (0.5 ant equivalents) (experiment 1) or to Dufour's gland extract ofworkers (experiment 2). In both experiments, gland extracts were prepared in dichloromethane (DCM) and DCMserved as the corresponding solvent control. Numbers in bars represent the number of ants selecting a test stimulus and numbers in white inset boxes represent the number of non-responding ants. The asterisks indicate a significant preference for a test stimulus (Pearson's χ^2 tests: P<0.001; n.s., P>0.05).**

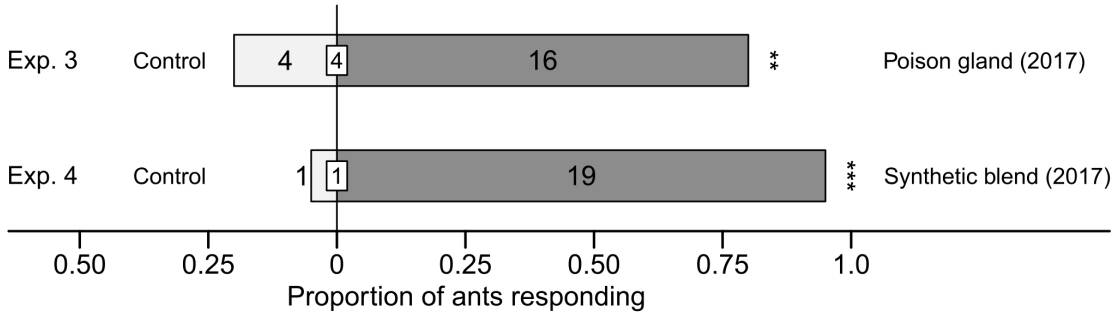

Figure 2.3. Proportion of *Camponotus modoc* worker ants responding in two-choice Y-tube olfactometers to poison gland extract ofworker ants (0.5 ant equivalents) (experiment 3) and to a synthetic blend ofcandidate poison gland pheromone components (experiment 4; see Table 2.1). In both experiments, treatment stimuli were presented in dichloromethane (DCM) andDCM served as the corresponding solvent control. Numbers in bars represent the number ofants selecting a test stimulus, and numbers inwhite inset boxes represent the number ofnon-responding ants. The asterisks indicate a significant preference for a test stimulus (Pearson's χ^2 tests; **0.001<P<0.01, ***P<0.001).

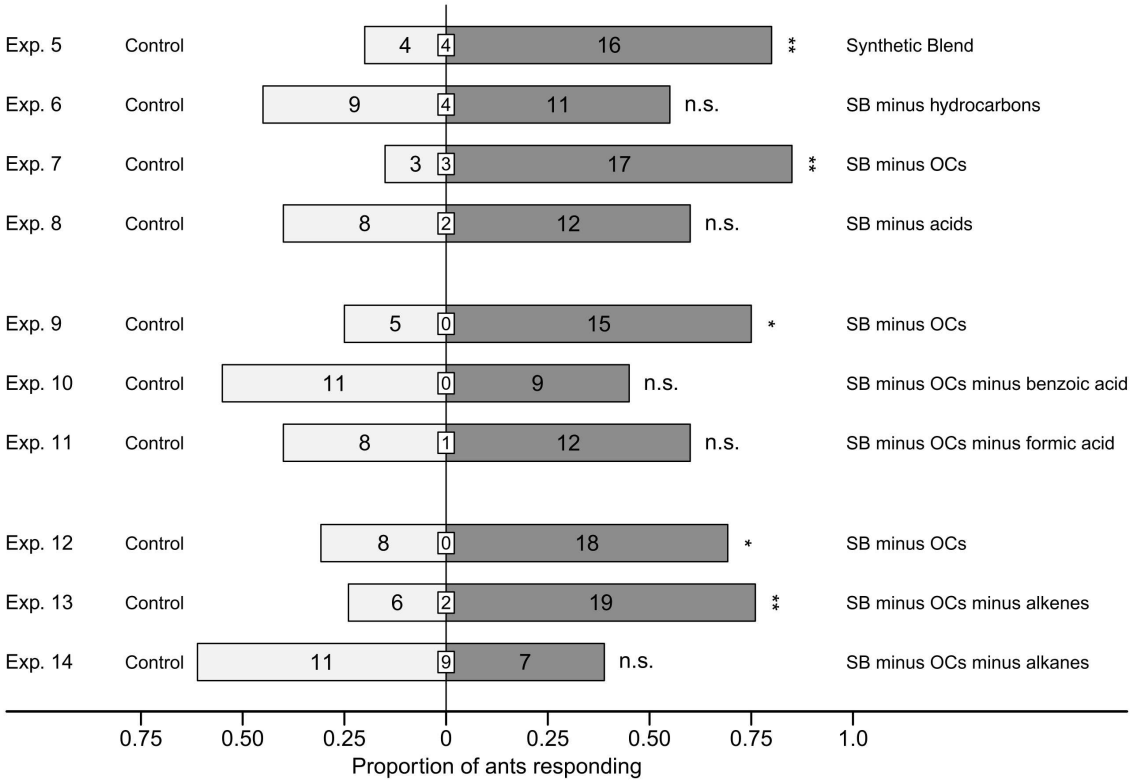

Figure 2.4. **Proportion of *Camponotus modoc* worker ants responding in two-choice Y-tube olfactometers to the complete synthetic blend (SB) of candidate alarm pheromone components (see Table 2.1 for blend composition) and to partial blends lacking certain groups of organic compounds (OCs means oxygenated compounds, i.e., hexadecan-1-ol, hexadecyl formate, and hexadecyl acetate). In all experiments, treatment stimuli were presented in dichloromethane (DCM) and DCM served as the corresponding solvent control. Numbers in bars represent the number of ants selecting a test stimulus and numbers in white inset boxes represent the number of non- responding ants. The asterisks denote a significant preference for a test stimulus (Pearson's χ^2 tests: *0.01<P<0.05, **P<0.01).**

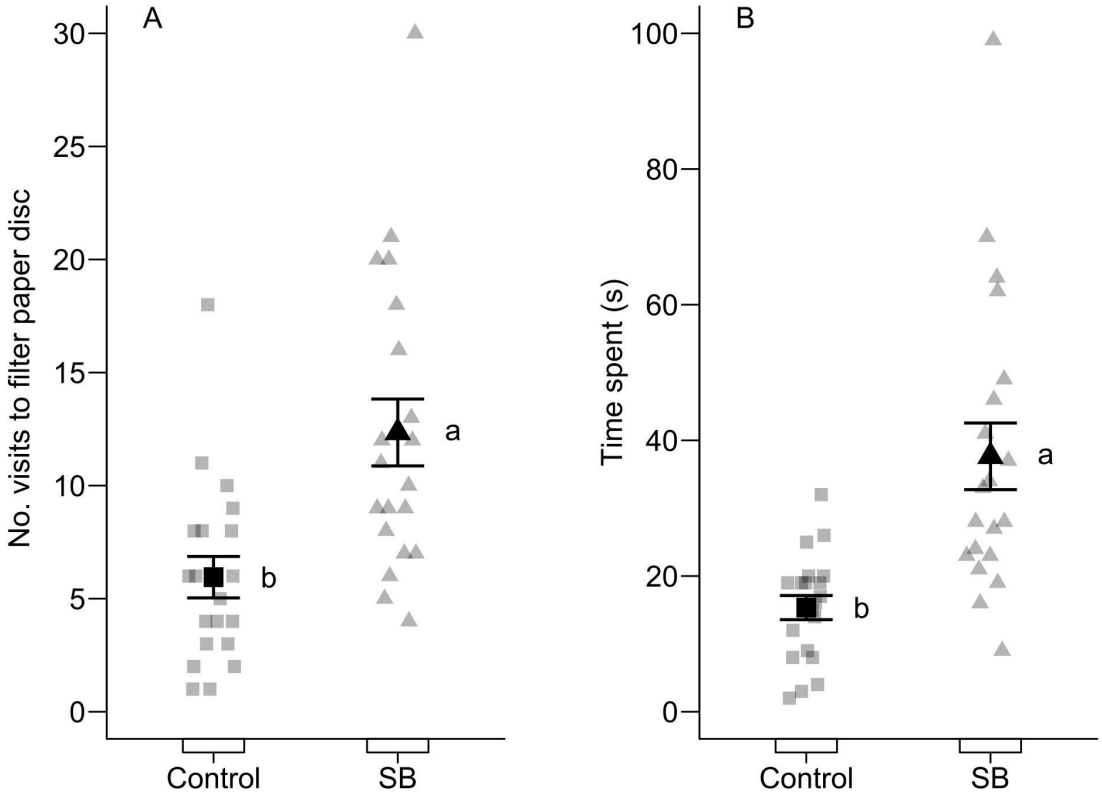

Figure 2.5. **Number of visits in arena bioassays by *Camponotus modoc* worker ants to two paper disc micro-locations and the time spent in those locations (see Figure 2.1 for setup). Micro- locations were treated with either a synthetic blend (SB) of candidate alarm recruitment pheromone components (see Table 2.1 for blend composition) or a solvent control. Grey symbols show the data of individual replicates and black symbols the experimental mean (± SE). The different letters near the means within a panel indicate significant treatment effects (paired t-tests: P<0.0007).**

Chapter 3.

Bi-modal alarm signals modulate responses to mono-modal alarm signals in *Camponotus modoc* carpenter ants

A similar version of this chapter has been submitted: Renyard, A., and Gries, G. (submitted). Bi-modal alarm signals modulate responses to mono-modal alarm signals in *Camponotus modoc* carpenter ants *Insect. Sci. INS-2024-01-050.*

3.1. Abstract

Carpenter ants, *Camponotus* spp., use alarm pheromone and substrate-borne vibrations to communicate the presence of threats to nestmates. The alarm pheromone reportedly attracts nestmates, whereas vibratory signals prompt ants to stand still (freeze) or run fast, helping predator evasion. When colonies are being attacked by a vertebrate predator, ants may engage in both pheromonal and vibratory signalling but behavioural responses of nestmates to bi-modal signals have not yet been investigated. Orientating towards signallers under vertebrate predator attack seems maladaptive and not beneficial to the colony. We tested the hypotheses (1) that vibratory alarm signals cause freezing, rapid running but not attraction of nestmates, and (2) that bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk. Laser Doppler vibrometry recordings revealed that the ants' vibratory signals readily propagate through ant nest lamellae, and thus quickly inform nestmates of perceived threats. With a speaker modified to record and deliver vibratory signals, we obtained drumming signals of distressed ants on a Douglas fir veneer, and bioassayed signal effects on ants in an arena with a suspended veneer floor. In response to playback of vibratory signals, ants ran rapidly, or froze, but did not approach the vibratory signals. Exposed to alarm pheromone, ants frequently visited the pheromone source. However, concurrently exposed to both alarm pheromone and vibratory signals, ants visited the pheromone source less often but spent more time 'frozen'. The ants' modulated responses to bi-modal signals seem adaptative but the reproductive fitness benefits are still to be quantified.

3.2. Introduction

In ants, colony defense is essential for survival. Colonies are generally dense and stationary aggregations of adult ants, brood, and food stores, and thus are lucrative targets for many predators, parasites, and ant competitors (Ayasse & Paxton, 2002; Abbot, 2022). These enemies exert strong selective pressures on colonies to have adequate defenses (Feldhaar, 2011; Abbot, 2022). Eusocial worker ants accrue fitness benefits through altruistic defensive behaviour that protect themselves and their colony, including their queen(s) and brood (Ayasse & Paxton, 2002; Abbot, 2022). For protection and defense, workers (*i*) build nest structures which are defensible and provide stable living conditions, (*ii*) use armaments such as stings, spines, toxins and biting mandibles, and (*iii*) communicate with nestmates to coordinate and optimize defense behavior (Nouvian & Breed, 2020).

Many ants respond to threats with coordinated defense (Dornhaus & Powell, 2010; Nouvian & Breed, 2020). *Formica rufa* wood ants engage in coordinated formic acid sprays to repel vertebrate enemies (Robinson & Stockdan, 2021), *Atta* leafcutter ants amass soldier ants to defend against army ants, *Nomamyrmex esenbeckii* (Powell & Clark, 2004), and myrmicine *Aphaenogaster cockerelli* ants simply abandon their nest (Smith & Haight, 2008). Single worker ants facing enemies flee, ignore the threat, become aggressive, or alarm-signal (Jelley & Moreau, 2023). Signal recipients respond by lowering their thresholds for aggression, fleeing, attraction, and locomotory changes (Hölldobler & Wilson, 1990; Dornhaus & Powell, 2010), and by more accurately recognizing nestmates (Rossi et al., 2019). The type of defensive behavior varies with the size of workers (Nowbahari et al., 1999; Parmentier et al., 2015), their age (Norman et al., 2014), past experience (Van Wilgenburg et al., 2010; Norman et al., 2014), and morphology (Powell, 2008; Huang, 2010). Groups of ants facing enemies modulate their defensive responses in relation to social context (Sakata & Katayama; Batchelor & Briffa, 2011; Chapman et al., 2011) and threat level (Scharf et al., 2011).

Ants coordinate defense through pheromonal and vibratory signals (Hölldobler & Wilson, 1990; Hunt & Richard, 2013). Volatile alarm pheromones readily disseminate, and substrate-borne vibratory signals quickly propagate through substrates, thus facilitating rapid information conveyance (Verheggen et al., 2010; Hunt & Richard, 2013). Distressed worker ants may discharge both alarm pheromone and defense chemicals,

with the latter possibly also having a signal function (Verheggen et al., 2010; Touchard et al., 2016). Alarm pheromones attract nestmates, incite aggression, or signal to flee (Hölldobler & Wilson, 1990). Hexanal and 1-hexanol emitted by weaver ants, *Oecophylla longinoda,* alert and attract nestmates, whereas 2-butyl-2-octenal and 3-undecanone mark enemies for attack (Bradshaw et al., 1975, 1979), and 4-methyl-3-heptanone and 4-methyl-3-heptanol emitted by clonal raider ants, *Ooceraea biroi*, signal nestmates to flee. Ant alarm pheromones have been well investigated but vibratory alarm signals for nest defense have hardly been studied (Golden & Hill, 2016). Ants cannot sense airborne sound but can perceive substrate-borne vibrations that are generated by stridulating, scraping and drumming (Hunt & Richard, 2013). Ants that are fighting or restrained (Markl, 1965; Stuart and Bell, 1980; Golden and Hill, 2016) produce stridulatory signals that attract nestmates (Markl, 1965; Roces et al., 1993b). Distressed workers of *Camponotus* carpenter ants produce drumming signals (Markl & Fuchs, 1972; Bota et al., 2022). Drumming signals that were experimentally engineered – as opposed to recorded from ants – and were input on plastic surfaces failed to attract ants but increased their running speed or, instead, stopped their locomotion, inducing a 'freezing response' (Markl & Fuchs, 1972; Fuchs, 1976).

Bi-modal alarm communication entailing pheromonal and vibratory signals has rarely been studied in ants. Distressed leafcutter ants, *Atta cephalotes*, release alarm pheromone and produce stridulatory sound which – in combination – attract conspecifics, as shown by experimentally rendering either communication modality dysfunctional. Foraging workers of the leafcutter ants *A. cephalotes* and *A. sexdens* orient to vibrating paths (Roces et al., 1993a; Roces & Hölldobler, 1996; Hager et al., 2017) but only in the absence of the alarm pheromone component citral (Hager et al., 2017). Foraging leafcutter ants prefer trail pheromone to vibratory signals for orientation but respond the best to a bi-modal signal complex (Hölldobler & Roces, 2001). Lastly, vibratory signals of *Camponotus* carpenter ants reduced the ants' threshold for aggressive responses to alarm pheromone (reported as an unpublished observation in Hölldobler, 1999).

The western carpenter ant, *Camponotus modoc*, is common in coniferous forests along the west coast of North America (Hansen & Klotz, 2005). Ants excavate galleries in trees, logs and stumps of conifers (Hansen & Akre, 1985), in the process often generating thin wooden lamellae that are perfect resonance bodies for vibratory

communication signals. Nests of carpenter ants face predation primarily by woodpeckers (Raley & Aubry, 2006; Gyug et al., 2014) and bears (Noyce et al., 1997; Swenson et al., 1999), with adult carpenter ants making up a large proportion (> 50%) of a pileated woodpecker's diet (Raley & Aubry, 2006). In response to threats, worker ants spray alarm pheromone that attracts nestmates (Renyard et al., 2020) and/or engage in drumming (A.R pers. obs.), presumably to produce vibratory signals. Behavioural responses to vibratory signals reported in other *Camponotus* spp. such as remaining still ('freezing') or rapid running (Markl & Fuchs, 1972; Fuchs, 1976), could be anti-predator responses (Dornhaus & Powell, 2010; Sakai, 2021). Rapid running enables predator evasion, and freezing renders ants invisible to predators that rely on prey motion for prey detection (Dornhaus & Powell, 2010; Sakai, 2021). Presented in isolation, the behavioural effect elicited by alarm pheromone (nestmate attraction) contrasts the effects of vibratory signals (freezing and rapid running of nestmates), and appears inconsistent. Orienting towards signallers under vertebrate predator attack seems maladaptive and not beneficial to the colony. Ants exposed to bi-modal signals (alarm pheromone and vibratory drumming) may modulate their behavioural responses that they typically exhibit when exposed to a mono-modal signal. We tested the hypotheses (1) that vibratory alarm signals cause freezing, rapid running but not attraction of nestmates, and (2) that bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk.

3.3. Materials and methods

3.3.1. Collection and maintenance of ants

We obtained and reared ant colonies as previously described (Renyard et al., 2019) with slight modification. Briefly, we collected ant nests from conifer forests near Squamish BC. Using a chainsaw, nests were cut from infested logs, placed in large bins (64 × 79 × 117 cm), and maintained in an outdoor undercover area on the Burnaby campus of Simon Fraser University, where they experienced natural light and weather cycles throughout the year. The bins were connected with Tygon® tubing (2.54 cm diam.) and barbed plumbing connectors (2.54 cm diam.) to glass containers (30.5 × 26 × 50.8 cm) that served as foraging arenas provisioned with apples, dead cockroaches, and 20% sugar water *ad libitum*. The upper inner walls of bins and containers were coated

with an equal mix of Vaseline (Unilever, London, UK) and paraffin oil (Anachemia, Lachine, QC, CA) to prevent ant escape. Bins and containers had lids with mesh covered holes to allow air exchange.

3.3.2. Hypothesis 1: Vibratory alarm signals cause freezing, rapid-running but not attraction of nestmates

Laser Doppler recordings of ant drumming on ant nest lamellae

Recordings and bioassays of vibratory signals were run in a dedicated room with wall-mounted acoustic panels to minimize sound reflections. All recording instruments were placed on a 1-ton concrete table to prevent vibratory background noise. Recording instruments were connected to computers in a separate room.

To record vibratory drumming signals produced by distressed ants, ants were placed on suspended wooden lamellae excised from ant nests, and were prompted to drum by poking them with a bamboo stake or a puff of air. Drumming signals were recorded using a single-point laser Doppler vibrometer (LDV; Polytech GmbH, DE), consisting of a VIB-A-510 illumination module and an OFV 534 compact sensor head connected to an OFV-2500 vibrometer controller. Vibrometry data were recorded using a Polytech VIB-E-220 Data Acquisition System and saved with Polytech VIBSOFT 4.7 (all products of Polytec Inc., Irvine, CA).

Recording ant drumming signals for behavioural responses

To obtain recordings of ant vibratory signals (see above) for behavioural responses of ants in bioassays, a veneer strip (2.5 × 61 × 0.05 cm) of Douglas fir, *Pseudotsuga menziesii* (Windsor Plywood, Langley, BC, CA) – comparable to ant nest lamellae – was suspended between two metal stands. To record signals, we used a modified speaker (Pyle PDMW5 5” midwoofer, 200W, 0.08–7kHz; Fig. 3.1a), with a metal wire (33 × 0.1 cm) glued to the speaker’s cone which, in turn, was secured by a retort ring clamp to the metal stand. The tip of the wire was brought into contact with the veneer such that the speaker’s cone remained in its neutral position for recordings. In preparation for recordings, ants were collected from two laboratory nests and held in plastic containers (24.4 × 12.7 × 8.9 cm) fitted with a test tube (10 mL) filled halfway with water and plugged with a cotton ball. To initiate recordings, individual ants (n = 7) were introduced onto the veneer with a bamboo stick and poked to induce drumming.

Drumming signals were recorded via the speaker using the software program Audacity (2.4.2). During recordings, the laser was focussed on the veneer center to monitor the velocity (mm/s) of signals for subsequent editing of the audio file (see below). All recordings were combined in a single audio file and looped to enable continuous playback in behavioural bioassays. During bioassays, the amplitude of playback input signals was adjusted to match that measured during signal recordings.

General protocol of behavioural bioassays

Behavioural experiments were run in a plexiglass arena (61 × 30.5 × 6 cm; Fig. 3.1b), with plexiglass blocks (5 × 3 × 5 cm) in each corner and along walls supporting a Douglas fir veneer (61 × 30.5 × 0.05 cm) which was affixed to arena walls with a 19-mm Scotch® tape (3M Company, Maplewood, MN, USA) to prevent warping. Two speakers were placed on opposite sides of the arena and secured to metal stands with their wire tips touching the veneer. A pencil line circumscribing each wire tip served as a reference in bioassays. The arena walls were coated with an equal mix of Vaseline and mineral oil to prevent ant escape.

For behavioral experiments, worker ants were collected from six laboratory colonies. To collect ants for a bioassay replicate, the Nalgene tubing inter-connecting a nesting bin and a foraging arena (see above) was detached from the arena, and an uncapped 15-mL Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA) – with a 0.7-cm-diameter hole cut in its tip – was inserted. As soon as five ants had entered the Falcon tube, it was removed, capped, and its open tip was plugged with a cotton ball.

To commence a bioassay, the Falcon tube was placed into the bioassay arena such that its tapered tip laid flush with the arena floor and was equidistant to each of the two test stimuli. We then removed the cotton plug from the tube's tip, allowing the ants 10 min (Exp. 1), or 2.5 min (Exp. 2), to 'calmly' exit the tube and walk into the arena. Once the first ant had entered the arena, we video-recorded (Canon Rebel t3i DSLR camera) the ants' behavior 5 min before, and 5 min during, the presentation of alarm stimuli. If no ants exited the tube, the replicate was excluded from analyses, and a new group of ants from the same nest was bioassayed instead. We used a new piece of veneer for each replicate, and cleaned both the arena and metal wires with hexane and ethanol between replicates.

Behavioural responses of ants to vibratory signals

We bioassayed responses of ants to vibratory drumming signals by playing back the looped audio file through one of the two speakers. The speaker was selected by stratified random assignment (Thompson, 2012), ensuring equal representation of either speaker for signal playbacks. Prior to the onset of an experimental replicate, we reviewed the quality of the playback using the LDV to make sure that the amplitude of playback signals matched that of signals recorded from ants.

3.3.3. Hypothesis 2: Bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk

In arena bioassays (Fig. 3.1), alarm pheromone and vibratory signals were tested singly, and in combination, for their effects on behavioral responses of ants. The synthetic alarm pheromone blend was dissolved in dichloromethane (DCM), and was tested at 0.5 (10-μL) ant equivalents [undecane (10 ng), tridecane (5 ng), pentadecane (1.5 ng), heptadecane (0.5 ng) (*Z*)-7-pentadecene (0.5 ng), (*Z*)-8-heptadecene (0.5 ng), (*Z*)-7-heptadecene (0.5 ng), hexadecan-1-ol (10 ng), hexadecyl formate (15 ng), hexadecyl acetate (1 ng), benzoic acid (6 ng), formic acid (5 μg)] per replicate (Renyard et al., 2020). The 10-μL pheromone blend and the corresponding 10-μL DCM control were applied to the wire tip of the treatment and the control speaker, respectively, where it made contact with the veneer. Both speakers were connected to a computer but remained silent throughout pheromone-only bioassays. The ants' responses to vibratory signals were tested as described above, except that the 10-μL DCM control was applied to the wire tip of both speakers before playback recordings were started. For testing the combined effect of alarm pheromone and vibratory signals, the 10-μL pheromone blend and the 10-μL DCM control were applied to the wire tip of treatment and control speakers, respectively, after which the treatment speaker was switched on playing back the looped audio file, with the control speaker remaining silent throughout the bioassay.

3.3.4. Analyses of video data

We scored video data in QuickTime Player (V10.5) and FIJI (V2.9.0). A 2.5-min interval of the video before, and during, the presentation of the test stimulus was analyzed to quantify attraction and freezing behaviour (i.e., no locomotion, and neither grooming nor trophallaxis). For each replicate, the total time an ant spent 'frozen' was summed up and averaged over all ants. For attraction, all visits by ants to the circles circumscribing the metal wire tips were counted. To determine the ants' running speed, we converted videos to Audio Video Interleave (AVI) files, and used the TrackMate (Ershov et al., 2022) plugin in FIJI to analyze a 15-s clip before and during the presentation of the test stimulus. Using the Hessian detector, we adjusted the object size (object diam XY: 30 pixels; diam Z: 16 pixels) and quality threshold to detect any ant moving on the veneer floor. We determined the mean running speed for each ant who had forward motion during the 15-s intervals, choosing her longest continuous path with consistent forward-movement – created by the program's 'tracker'– by converting the speed of the ant's pixels per frame to cm/s, and then averaging data among all ants in each replicate.

3.3.5. Statistical analyses

Data were analyzed and graphics were prepared using R (v. 4.2.2) and R studio (v. 2022.07.1+554)(R Core Team, 2022). We processed data using the tidyverse packages (Wickham et al., 2019) and plyr function (Wickham, 2011). The glmmTMB package (Brooks et al., 2019) was used to fit generalized linear mixed models (GLMMs), and model fit was inspected using the DHARMa package (Hartig, 2022). We used the emmeans package (Lenth, 2023) to calculate estimated marginal means and 95% confidence intervals. Graphics were produced using ggplot2 (Wickham, 2016), assembled using patchwork (Pedersen, 2023), and final figure editing was completed in Inkscape (v. 1.0.2).

We analyzed our data (Renyard and Gries, 2024) using GLMMs. To account for multiple comparisons performed within a replicate, all models were fit with 'replicate' as a random intercept. For analysis of data collected for testing hypothesis 1, we fit the mean time spent frozen, and running speed, as response variables with 'Before' and 'During' stimulus exposure as a predictor variable using a tweedie distribution. The number of

visits by ants to speaker wires was fit as an interaction between 'Before' and 'During' stimulus presentation, and side of stimulus was fit with a Poisson distribution. For analysis of data collected for testing Hypothesis 2, we fit mean time spent frozen, and running speed, as response variables with an interaction between 'Before' and 'During' stimulus exposure, and treatment stimulus, as predictors, with a tweedie and gamma distribution, respectively. Number of visits was fit as a function of treatment, side of stimulus, and 'Before' and 'During' stimulus presentation, and three two-way interactions between each categorical predictor using a negative binomial distribution. We selected error distributions that were appropriate for data types and that offered the best model fit. Significance of our predictors or interactions were evaluated using a likelihood ratio test (LRT). We made specified *a priori* contrasts within treatments and timepoints, and across treatments, using Tukey adjusted pairwise comparisons.

3.4. Results

3.4.1. Hypothesis 1: Vibratory alarm signals cause freezing, rapid running but not attraction of nestmates

Laser Doppler recordings of ant drumming

Ant drumming signals readily propagated through wooden lamellae excised from ant nests (Fig. 3.2; Fig. A1). Signals occurred in clusters and sometimes in triplets.

Behavioural responses of ants to vibratory signals

Exposed to vibratory signals, ants spent more time 'frozen' (LRT: $\chi^2 = 21.76$, d. f. = 1, $p < 0.0001$; Fig. 3.3a; Table A1), and ran faster (LRT: $\chi^2 = 13.44$, d. f. = 1, $p = 0.0002$; Fig. 3.3b; Table A1) than in the absence of these signals. Ants visited the circle circumscribing the wire tip of either speaker equally often before, and during, the presentation of vibratory signals (Table A1). Neither treatment side nor interaction between treatment side and visits, before and during vibratory signal exposure, were significant predictors in the statistical model (LRT: side of treatment: $\chi^2 = 3.68$, d. f. = 2, $p = 0.16$; interaction: $\chi^2 = 0.98$, d. f. = 1, $p = 0.32$). However, the number of visits, before and during the presentation of vibratory signals, was a significant predictor (LRT: $\chi^2 = 14.76$, d. f. = 2, $p = 0.0006$), indicating that visits to either speaker decreased after vibratory signals commenced.

3.4.2. Hypothesis 2: Bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk

Time spent 'frozen' by ants differed in response to the type of stimulus tested (alarm pheromone, vibratory signal, both) (Fig. 3.4a). Stimulus type, the ants' responses before and during stimulus presentation, and the interaction between stimulus type and the ants' responses before and during stimulus presentation, were all significant predictors in the model (LRT: stimulus type: $\chi^2 = 16.69$, d. f. = 4, $p = 0.002$; responses before and during stimulus presentation: $\chi^2 = 43.34$, d. f. = 3, $p < 0.0001$; interaction: $\chi^2 = 7.88$, d. f. = 2, $p = 0.019$). The vibratory signals alone, and in combination with alarm pheromone, each elicited longer freezing responses than the alarm pheromone alone ($p < 0.05$; Table A2). The combination of vibratory signals and alarm pheromone elicited freezing responses twice as long as the vibratory signals alone but this 2-fold difference in time spent frozen was statistically not significant (Table A2).

Running speed also differed in response to the stimulus type tested (Fig. 3.4b). Stimulus type, the ants' responses before and during stimulus presentation, and the interaction between stimulus type and the responses before and during stimulus presentation, were all significant predictors in the model (stimulus type: $\chi^2 = 29.64$, d. f. = 4, $p < 0.0001$; responses before and during stimulus presentation: $\chi^2 = 43.83$; d. f. = 3, $p < 0.0001$; interaction: $\chi^2 = 13.68$, d. f. = 2, $p = 0.001$). Vibratory signals alone, and in combination with alarm pheromone, each prompted ants to run faster than the alarm pheromone ($p < 0.05$; Table A3). Numerically, but not statistically, ants ran faster in response to vibratory signals than in response vibratory signals and alarm pheromone (Table A3).

The attraction of ants to test stimuli varied with stimulus type (Fig. 3.4c). Alarm pheromone, the ants' responses before and during stimulus presentation, treatment side, and the interaction between stimulus type and responses before and during stimulus presentation, were all significant predictors in the model (stimulus type: $\chi^2 = 15.51$, d. f. = 6, $p = 0.017$; responses before and during stimulus presentation: $\chi^2 = 33.47$, d. f. = 4, $p < 0.0001$; treatment side: $\chi^2 = 33.56$, d. f. = 4, $p < 0.0001$). Statistically significant were the interaction terms between the ants' responses before and during stimulus presentation and the number of visits to speakers ($\chi^2 = 11.40$, d. f. = 1, p =

0.0007), and between the vibratory signal type and number of visits to speakers (χ^2 = 8.46, d. f = 2, p = 0.015), whereas the interaction between stimulus type and the ants' responses before and during stimulus presentation was not significant (χ^2 = 4.86, d. f = 2, p = 0.088). Before the presentation of test stimuli, there was a numerically weak but statistically significant difference ($p < 0.05$; Table A4) in the number of visits to the treatment speaker in the alarm pheromone experiment but not in the experiments that tested the effects of vibratory signals alone or in combination with the alarm pheromone ($p > 0.05$; Table A4). Exposed to test stimuli, ants visited the treatment speaker presenting vibratory signals, alarm pheromone, or both, significantly more often than the control speaker lacking any of these stimuli ($p < 0.05$; Table A4). During stimulus exposure across experiments, ants visited the speakers presenting vibratory signals alone or in combination with alarm pheromone significantly less than the speaker with just the alarm pheromone treatment ($p < 0.05$ each; Table A4), with the former two stimulus types generating similar numbers of ant visits ($p > 0.05$; Table A4).

3.5. Discussion

Our data support the hypothesis that vibratory alarm signals prompt nestmates to run faster, or to remain still, but do not attract them. Our data also support the hypothesis that bi-modal alarm signals modulate the ants' responses to mono-modal alarm signals, thereby likely reducing predation risk.

Worker ants – placed on wooden lamellae excised from ant nests, and agitated by a stick or puff of air – produced distinct vibratory signals (Fig. 3.2) by slamming their abdominal tip or head against lamellae. As indicated by laser Doppler vibrometry recordings, the signals readily propagated through the lamellae and would be sensed by nestmates residing on the same lamella, thereby alerting them to a predatory threat possibly already present on the nest. In response to playback recordings of these signals, ants stood still or doubled their running speed but hardly ever approached the source of vibratory signals, as we predicted and as was previously reported in *Camponotus* spp. (Markl & Fuchs, 1972; Fuchs, 1976).

All of these behavioral responses seem adaptive. Rapid running and path complexity or tortuosity are behavioral tactics of ants (Fuchs, 1976; Markl & Fuchs, 1972; Angilletta et al., 2008) that may improve the likelihood of escape. Running is

comparable to the escape tactics of arboreal *Cephalotes atratus* ants that jump or drop off branches and aerially glide to other plant parts (Weber, 1957; Yanoviak et al., 2005), thus reducing predation (Dudley et al., 2007). Similarly, *Myrmecina graminicola* ants detecting a threat while being on a slope curl up their body and roll away (Grasso et al., 2020). The tactic of freezing is reported in diverse insect taxa (Sakai, 2021) and in several species of ants (Markl & Fuchs, 1972; Fuchs, 1976; Grasso et al., 2020; this study). Freezing is diametrically opposite to running but likely has also a predator avoidance function (Dornhaus & Powell, 2010). Freezing may render ants invisible to predators that rely primarily on prey motion for prey detection (Dornhaus & Powell, 2010; Sakai, 2021).

To not approach a nestmate producing vibratory alarm signals (Figure 3; Markl & Fuchs, 1972; Fuchs, 1976) seems adaptive in that ants in clusters or aggregations would draw the attention of the vertebrate predator, and make easy and profitable targets. Even the concerted effort of all aggregated ants would not be sufficient to defend the nest against large predators such as bears or woodpeckers.

Presented as a mono-modal signal, synthetic alarm pheromone attracted *C. modoc* workers (Fig. 3.4), as previously shown (Renyard et al., 2020). Alarm pheromone components are often volatile (Hölldobler & Wilson, 1990; Morgan, 2008), disseminate readily, and thus quickly inform nestmates about threats. However, approaching a pheromone-signaling nestmate under attack by a vertebrate predator seems maladaptive (see above) as it would increase the risk of responding ants of being preyed upon without engendering any benefits to the colony. The alarm recruitment pheromone of *C. modoc* contains constituents that serve dual functions as pheromone components and defense chemicals, and may primarily be discharged in a context other than nest defense against vertebrate predators. Alarm pheromone sprays do not readily spread through the nest's interior and are likely more effective outside nests. Formic acid as a spray constituent is toxic to both invertebrate enemies (e.g., other ants; Chen et al., 2012) and to vertebrate enemies, (e.g., birds, albeit only with prolonged exposure; Bennett et al., 1996), and undecane helps spread formic acid over enemy cuticle (Leclercq et al., 2000). The alarm pheromone discharged may also be intended less to attract nestmates than to heighten their aggression towards vertebrate and invertebrate predators (Hölldobler & Wilson, 1990; Baracchi et al., 2021). Lastly, ants may simply spray alarm pheromone as a 'last-ditch' effort to escape predation.

The bi-modal signal complex of alarm pheromone and vibration elicited behavioral responses by ants contrasting those to mono-modal alarm pheromone or vibratory signals. In the presence of both signal modalities, ants visited the pheromone source – the pheromone-baited speaker – less frequently, indicating that vibratory signals down-regulated attraction of nestmates to pheromone. Moreover, pheromonal and vibratory signals in combination prompted longer 'freezing' bouts in signal recipients than pheromonal or vibratory signals alone, and faster running speed than pheromone alone. If we accept the premise that attraction to pheromone is maladaptive in the face of a nest-attacking predator (see above), and that freezing and rapid running are effective predator evasion tactics (Dornhaus & Powell, 2010; Sakai, 2021), then pheromonal and vibratory signals in combination have complementary effects that are beneficial to responding ants, essentially reducing their risk of predation.

The type of defensive behavior by ants is dependent upon many factors. Defensive behavior of single ants is modulated by their body size (Nowbahari et al., 1999; Parmentier et al., 2015), age (Norman et al., 2014), experience (Van Wilgenburg et al., 2010; Norman et al., 2014), morphology (Powell, 2008; Huang, 2010), and physiological status (Sasaki et al., 2014; Pokorny et al., 2020), whereas defensive behavior at the colony level is dependent upon the presence of nestmates (Sakata & Katayama, 2001; Batchelor & Briffa, 2011; Chapman et al., 2011) and the threat level (Scharf et al., 2011). For examples, *Platythyrea punctata* ants age-dependently both produce and respond to alarm pheromone, and *Temnothorax rugatulus* worker ants avoid alarm pheromone in unfamiliar terrain but approach pheromone at their nesting site (Sasaki et al., 2014; Pokorny et al., 2020). We provide a first insight into bi-modal communication of carpenter ants but the distinct cues and contexts that trigger specific pheromonal, vibratory or bi-modal communication are still not well understood. Moreover, the reproductive fitness benefits to the colony accrued by producing, or responding to, mono- or bi-modal communication signals are yet to be experimentally quantified.

3.6. Data Availability Statement

Data are available from Mendeley Data and can be accessed at: DOI: 10.17632/hc33hbhg8t.1 (Renyard & Gries, 2024).

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3.9. Tables

Table 3.1. Table 1: Hypotheses (H) tested with *Camponotus modoc* carpenter ants, alarm signals presented, number of bioassay replicates run (n), response criteria measured, and statistical comparisons of the ants' responses (*i*) 'Before' and 'During' signal presentations, and (*ii*) to treatment and control signals 'Before' or 'During' signal presentations.

Exp. #	n	Alarm signals	Response criteria measured	Statistical comparisons
(H1) Vibratory alarm signals cause freezing, rapid running but not attraction of nestmates				
1	11	Vibration	Time spent 'frozen'[1]	Before *vs* During
			Running speed	Before *vs* During
			# Visits to speaker wires	Before: control *vs* treatment
				During: control *vs* treatment
(H2) Bi-modal alarm signals modulate responses to mono-modal alarm signals, thereby likely reducing predation risk				
2	9	Pheromone	Time spent 'frozen'	Before *vs* During
			Running speed	Before *vs* During
			# Visits to speaker wires	Before: control *vs* treatment
				During: control *vs* treatment
3	10	Vibration	Time spent 'frozen'	Before *vs* During
			Running speed	Before *vs* During
			# Visits to speaker wires	Before: control *vs* treatment
				During: control *vs* treatment
4	10	Combination	Time spent 'frozen'	Before *vs* During
			Running speed	Before *vs* During
			# Visits to speaker wires	Before: control *vs* treatment
				During: control *vs* treatment

[1]Frozen = No locomotion, and neither grooming nor trophalaxis behaviour

3.10. Figures

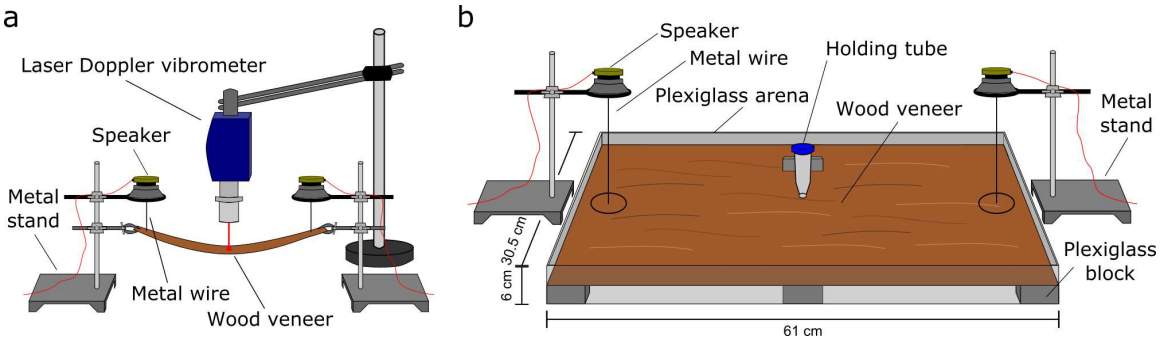

Figure 3.1. **Design of setup (a) for recording vibratory signals of *Camponotus modoc* carpenter ants, and (b) for bioassaying the ants' responses to vibratory signals and to alarm pheromone. (a) For recordings, a wooden strip of Douglas fir veneer was suspended by clamps between two metal stands. Two speakers were secured by retort ring clamps to metal stands, and a metal wire was glued to the speakers' cone and brought into contact with the veneer for recording vibratory drumming by ants. Vibrations were recorded on a computer using Audacity. A laser Doppler vibrometer was focussed on the veneer center to monitor the amplitude and propagation of ant vibratory signals. (b) A thin Douglas fir veneer was supported by plexiglass blocks in a plexiglass arena. Two speakers were secured to metal stands, and the tip of the metal wire attached to the speakers' cone was brought into contact with the veneer. A pencil line circumscribing the wire tip served as a reference for counting ant visits to a speaker. For each bioassay replicate, five ants entered the arena from a 15-mL Falcon (holding) tube with its tapered tip cut open. The behaviour of these ants was video-recorded 5 min before, and 5 min during, the presentation of vibratory signals, alarm pheromone, or both. Vibratory signals were played back through a speaker and synthetic alarm pheromone was pipetted to the tip of one or both speaker wires.**

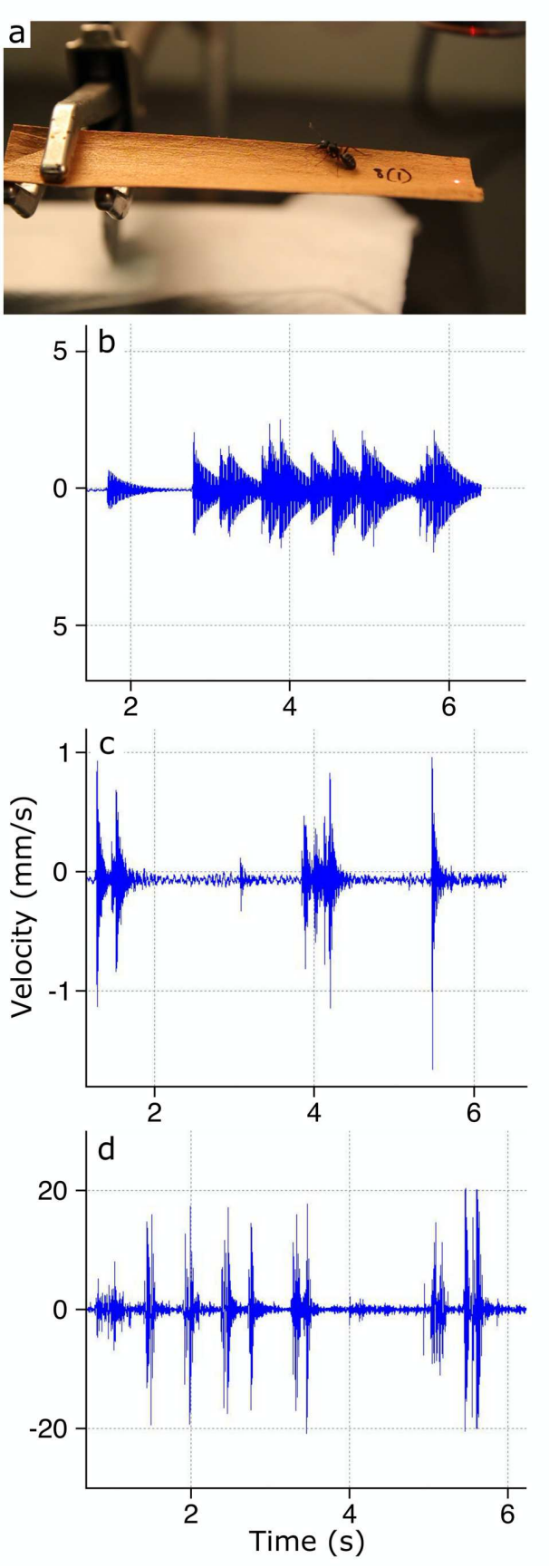
a
b
5
0
5
2
4
6
c
1
0
-1
Velocity (mm/s)
2
4
6
d
20
0
-20
2
4
6
Time (s)

Figure 3.2. **(a) Representative photograph of a worker carpenter ant, *Camponotus modoc,* on a thin wooden lamella excised from a carpenter ant nest, and (b–d) representative recordings of vibratory signals produced by ants and recorded by laser Doppler vibrometry. For recordings, a lamella (n = 2) was suspended by a metal clamp secured to a metal stand. The laser was focussed perpendicular to the lamella 5–16 cm apart from the clamp, and a worker ant was placed on the lamella, and prompted to produce vibratory signals by agitating it with a stick or a puff of air.**

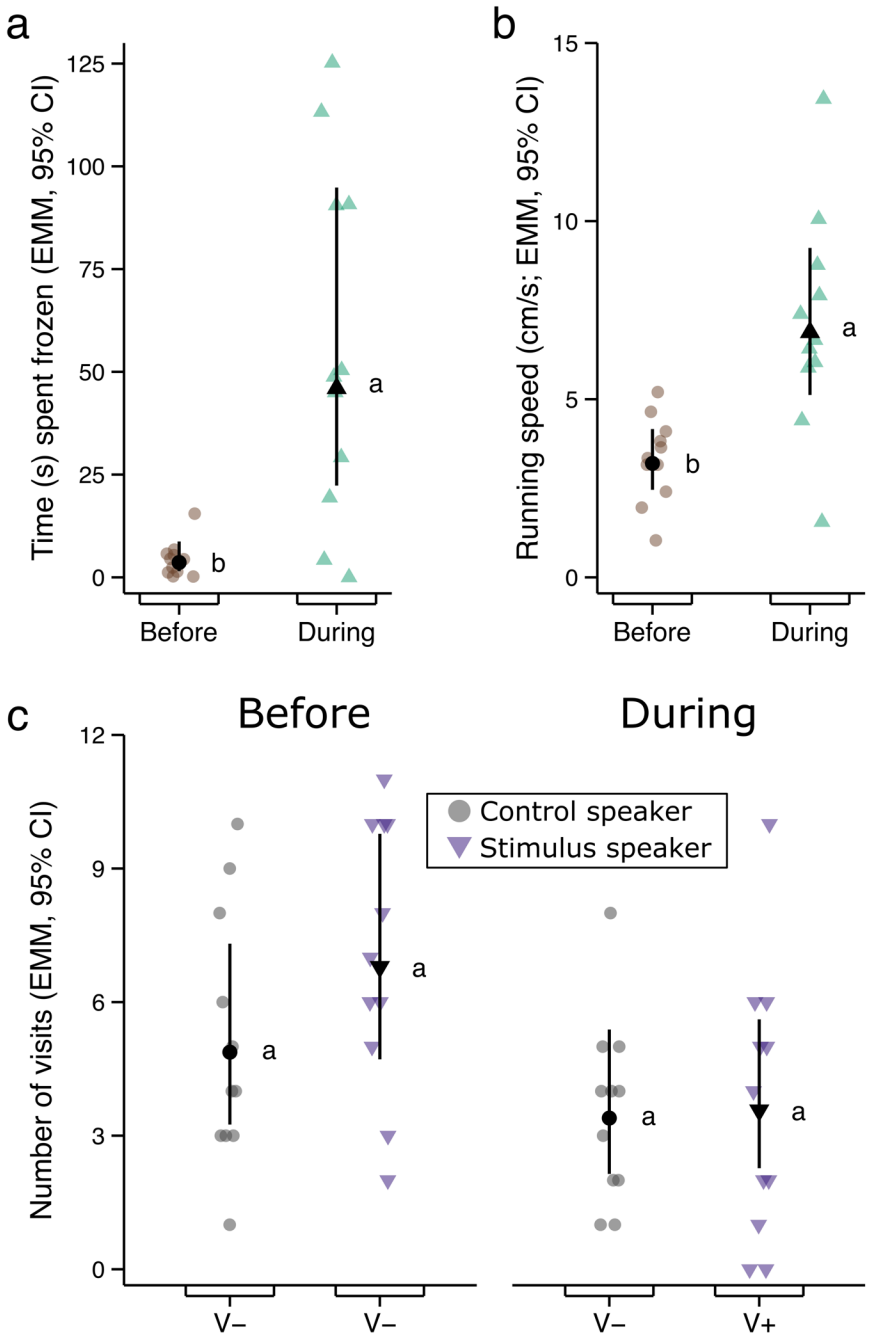
a
Time (s) spent frozen (EMM, 95% CI)
125
100
75
50
25
0
b
a
Before
During
b
Running speed (cm/s; EMM, 95% CI)
15
10
5
0
b
a
Before
During
c
Before
During
Number of visits (EMM, 95% CI)
12
9
6
3
0
Control speaker
Stimulus speaker
a
a
a
a
V−
V−
V−
V+

Figure 3.3. **Behavioral changes of *Camponotus modoc* worker ants in response to playback of vibratory *C. modoc* vibratory signals (Figs. 1b, 2). For each replicate (n = 11), the behavior of 5 worker ants was video-recorded 5 min before ('Before'), and 5 min during ('During'), the playback of vibratory signals. Time spent frozen (no locomotory behaviour) (a), running speed (b), and numbers of visits by ants to circles around speaker wire tips that played back vibratory signals (V+), or that remained silent (V–), were scored. Individual color symbols represent the data of a 5-ant group, and black symbols and whiskers represent back-transformed estimated marginal means (EMM) and 95% confidence intervals (CI). Different lower-case letters next to means indicate statistical differences in the ants' responses (Likelihood ratio test; $p < 0.05$). In subpanel c, stimulus speaker and the interaction between speaker and before and during stimulus were not significant predictors of ant behavior in our model (likelihood ratio test; $p > 0.05$) but before and during stimulus was a significant predictor (Likelihood ratio test; $p < 0.05$).**

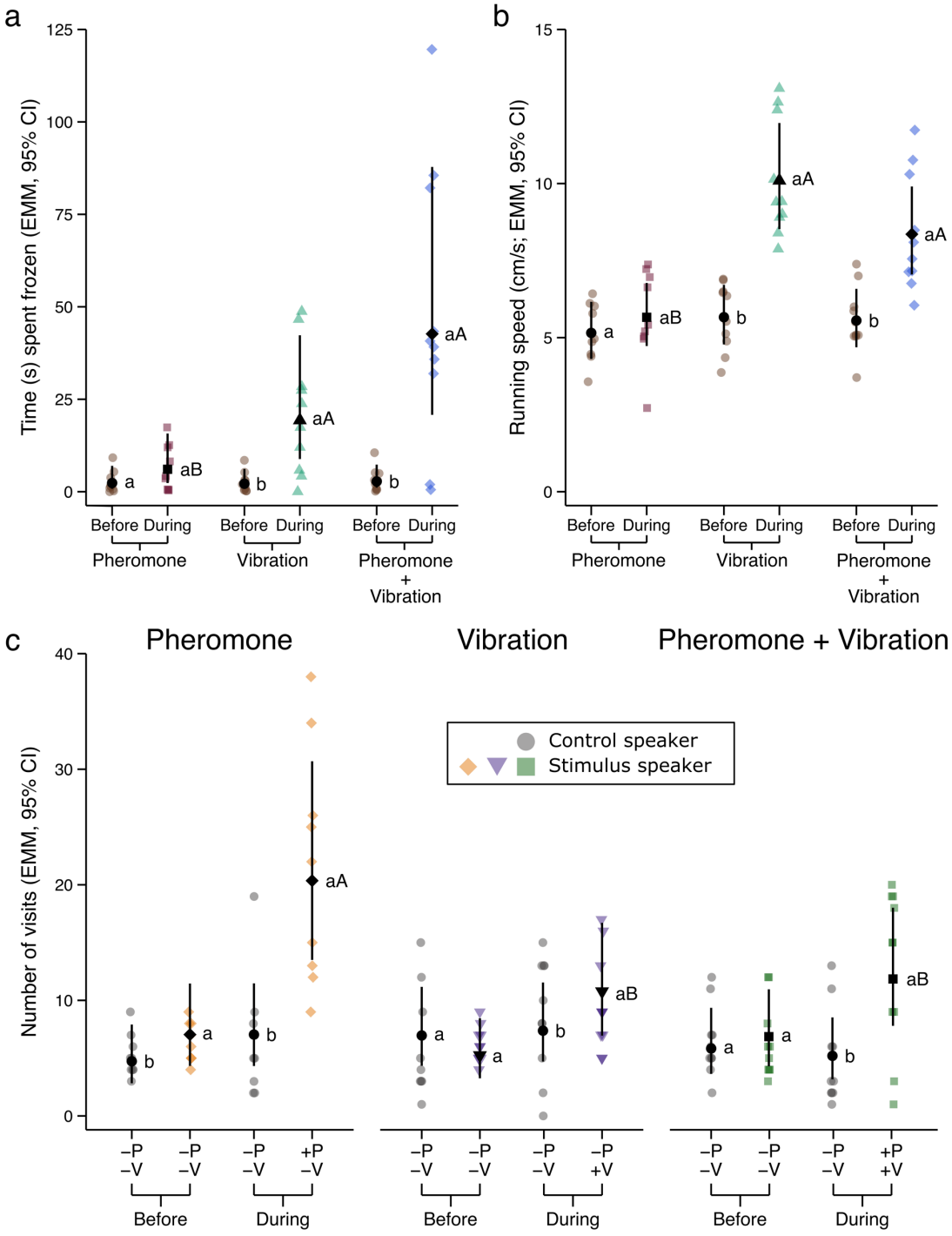
a
Time (s) spent frozen (EMM, 95% CI)
125
100
75
50
25
0
a
aB
b
aA
b
aA
Before During
Pheromone
Before During
Vibration
Before During
Pheromone + Vibration
b
Running speed (cm/s; EMM, 95% CI)
15
10
5
0
a
aB
b
aA
b
aA
Before During
Pheromone
Before During
Vibration
Before During
Pheromone + Vibration
c
Pheromone
Vibration
Pheromone + Vibration
Number of visits (EMM, 95% CI)
40
30
20
10
0
Control speaker
Stimulus speaker
b
a
b
aA
a
a
b
aB
a
a
b
aB
–P –V
–P –V
–P –V
+P –V
–P +V
Before
During

Figure 3.4. **Behavioral changes of *Camponotus modoc* worker ants when exposed to alarm pheromone (n = 9), vibratory signals (n = 10), or both (n = 10) (Figs. 1b). For each replicate, the behavior of five worker ants was video-recorded 5 min before ('Before'), and 5 min during ('During'), the presentation of test stimuli. The time spent frozen (no locomotory behaviour) (a), running speed (b), and the number of visits by ants to circles around speaker wire tips that played back vibratory signals (V+), or not (V–), and that received alarm pheromone (P+), or not (P–), were scored. Individual color symbols represent the data of a 5-ant group, and black symbols and whiskers represent back-transformed estimated marginal means (EMM) and 95% confidence intervals (CI). Within a treatment (alarm pheromone, vibratory signals, or both) different lower-case letters next to means indicate statistically different responses by ants in the periods 'Before' and 'During' stimulus exposure (a, b) or different numbers of ant visits to treatment and control speakers within the same period, either 'Before' or 'During' stimulus presentation. Different upper-case letters indicate statistically different responses by ants across treatments (Tukey adjusted $p < 0.05$). Note: in subpanel c (pheromone treatment), there was a numerically small, but statistically significant, difference in the number of ant visits to treatment and control speakers which presented identical stimuli. As this type of differential response to the treatment speaker did not occur in any other experiment [see subpanel c in Figure 3, and subpanel c in Figure 4 ('Vibration' and 'Pheromone + Vibration)], we trust that this bias response to the treatment speaker is coincidental.**

Chapter 4.

Floral and bird excreta semiochemicals attract western carpenter ants

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4.1. Abstract

Ant colonies have vast and diverse nutritional needs but forager ants have limited mobility to meet these needs. Forager ants would accrue significant energy savings if they were able to sense and orient toward odor plumes of both carbohydrate and protein food sources. Moreover, if worker ants, like other flightless insects, had reduced olfactory acuity, they would not recognize the specific odor signatures of diverse carbohydrate and protein sources, but they may be able to orient toward those odorants that are shared between (macronutrient) food sources. Using the Western carpenter ant, *Camponotus modoc*, as a model species, we tested the hypotheses that (1) food sources rich in carbohydrates (aphid honeydew, floral nectar) and rich in proteins (bird excreta, house mouse carrion, cow liver infested or not with fly maggots) all prompt long-distance, anemotactic attraction of worker ants, and (2) attraction of ants to plant inflorescences (fireweed, *Chamaenerion angustifolium*; thimbleberry, *Rubus parviflorus*; and hardhack, *Spiraea douglasii*) is mediated by shared floral odorants. In moving-air Y-tube olfactometer bioassays, ants were attracted to two of four carbohydrate sources (thimbleberry and fireweed), and one of four protein sources (bird excreta). Headspace volatiles of these three attractive sources were analyzed by gas chromatography-mass spectrometry, and synthetic odor blends of thimbleberry (7 components), fireweed (23 components), and bird excreta (38 components) were prepared. In Y-tube olfactometer bioassays, synthetic blends of thimbleberry and fireweed but not of bird excreta attracted ants, indicating that only the two floral blends contained all essential attractants. A blend of components shared between thimbleberry and fireweed was not attractive to ants. Our data support the conclusion that *C. modoc* worker ants can sense and orient toward

both carbohydrate and protein food sources. As ants were selective in their responses to carbohydrate and protein resources, it seems that they can discern between specific food odor profiles and that they have good, rather than poor, olfactory acuity.

4.2. Introduction

Ant colonies have vast nutritional needs. Foraging worker ants must meet not only their own nutritional needs but also those of their nestmates. Worker ants require primarily carbohydrates for energy, whereas the queen(s) and brood also require proteins for egg production and larval development, respectively (Markin, 1970; Sorensen and Vinson, 1981; Weeks et al., 2006). To meet these nutritional needs, ants engage in complex and diverse foraging activities. Ants obtain sugary honeydew excretions from hemipteran insects, hunt for insect prey, scavenge for deceased insects, feed on floral and extrafloral nectar as well as pollen, collect plant seeds, harvest plant foliage to cultivate mutualistic fungi, and acquire nutrients from animal excreta and carrion (Hölldobler and Wilson, 1990). The ants' foraging activities may alter biotic and abiotic characteristics of their habitat, including the plant community composition (Halaj et al., 1997; Macmahon et al., 2000; Swanson et al., 2019).

The means by which foraging ants locate food sources have rarely been studied (Knaden and Graham, 2016). Engaging in certain foraging patterns may increase the likelihood of locating food (Dornhaus and Powell, 2010) but sensing and responding to cues from food sources would make foraging more energy- efficient. Visual cues associated with insect prey seem to guide some foraging ants (Baroni Urbani et al., 1994; Beugnon et al., 2001). Olfactory resource cues guide many foraging insects (Cardé and Willis, 2008; Webster and Cardé, 2017) and – as shown in a few studies – also guide ants (e.g., Zhou et al., 2012; Fischer et al., 2015). Some ants learn to associate odors with food sources, and via trophallaxis pass on food odor information to nestmates (Dupuy et al., 2006; Provecho and Josens, 2009; Nelson et al., 2019; Oberhauser et al., 2019). In some cases, these associations can be learned quickly. For example, *L. niger* ants learn to associate sugar rewards after a single visit to a feeder (Czackzes and Kumar, 2020). Innate recognition of certain food odors would expedite the process of locating resources that are reliably present, whereas learned odors may help locate and exploit fleeting resources.

Foraging requires energy expenditures not only for the locomotory physical activity but also for the maintenance of those sensory receptors and nervous tissues that inform foraging activities (Niven and Laughlin, 2008; Dornhaus and Powell, 2010; Elgar et al., 2018). As flightless foragers, ants have limited mobility and would accrue significant energy savings for themselves and for the entire colony, if they were able to track the odor plume from all valuable resources and pinpoint their location from a distance. Specific ant taxa are known to respond to odor cues from specific resources, such as deceased insects (Buehlmann et al., 2014; see below), honeydew (Zhou et al., 2012; Fischer et al., 2015; see below), and floral nectar (Schiestl and Glaser, 2012; De Vega et al., 2014; see below) but to date no study has investigated whether conspecific ants are able to respond to odor cues from multiple macronutrient sources including those consisting of mainly carbohydrates and proteins. This ability would be adaptive because foragers must adjust their foraging activities and priorities in accordance with their colony's needs. When brood is present, they must collect not only more food but also more proteinaceous food (Cornelius and Grace, 1997; Dussutour and Simpson, 2008, 2009)

Protein-rich food sources such as insect prey, carrion, and animal excreta are often ephemeral. Challenged to locate them quickly, scavenging desert ants, *Cataglyphis fortis*, use olfaction to find deceased insects (Buehlmann et al., 2014), and the ponerine ant *Pachycondyla analis* and the formicine ant *Crematogaster scutellaris* exploit prey odor to locate termite and fig wasp prey, respectively (Schatz et al., 2003; Yusuf et al., 2014). As protein sources commonly release indole – which is a breakdown product of tryptophan and is an indicator of essential amino acid presence (Tomberlin et al., 2016) – many insects, including *C. fortis*, use indole as a generic semiochemical to locate protein sources (Chaudhury et al., 2015; Zito et al., 2015; Brodie et al., 2016, 2018; Cortez et al., 2017; Zhao et al., 2020). Similarly, workers of *C. fortis* are attracted to linoleic acid, a necromone indicative of deceased insects (Buehlmann et al., 2014).

Carbohydrates are vital to ant colony survival (Cook et al.,2010; Dussutour and Simpson, 2012; Bazazi et al., 2016; Arganda et al., 2017). Carbohydrates sought by ants originate mainly from floral and extrafloral nectar and sugary honeydew. Previously considered deleterious nectar thieves (Willmer et al., 2009), ants are increasingly documented as floral visitors and pollinators (De Vega et al., 2009; Czechowski et al., 2011; Luo et al., 2012; Ibarra- Isassi and Sendoya, 2016; Kuriakose et al., 2018; Del-

Claro et al., 2019; Delnevo et al., 2020). Yet, there are still only a few examples of ant attraction to honeydew or floral and leaf semiochemicals (message bearing chemicals). Workers of both the black garden ant, *Lasius niger*, and the red-imported fire ant, *Solenopsis invicta*, are attracted to honeydew excreted by aphids (Zhou et al., 2012; Fischer et al., 2015). Similarly, workers of the African weaver ant, *Oecophylla longinoda*, respond to leaf odors of cashew trees, *Anacardium occidentale*, and protect these trees from herbivores in exchange for extrafloral nectar rewards (Wanjiku et al., 2014). Some species of ants respond to herbivore-induced plant volatiles (Agrawal and Dublin-Thaler, 1998; Bruna et al., 2008; Schettino et al., 2017), while others are attracted to floral odors of specific plants (*Cytinus hypocistis, Chamorchis alpine*), serving as their exclusive pollinators (De Vega et al., 2009, 2014; Schiestl and Glaser, 2012).

If worker ants had reduced olfactory acuity, like other flightless insects (Neupert et al., 2020), they would not likely be able to recognize the specific odor profiles of diverse food sources but might still be able to locate them by responding to key odorants shared between these sources. For example, many inflorescences that provide essential carbohydrates to pollinators share linalool and α-pinene as floral attractants (Knudsen et al., 2006; Nicolson, 2011). Whether foraging ants respond to specific or generic carbohydrate semiochemicals has not yet been investigated.

Western carpenter ants, *Camponotus modoc*, as a model species in our study, are commonly found in coniferous forests along the west coast of North America (Hansen and Klotz, 2005). They forage on aphid honeydew (Tilles and Wood, 1982; Renyard et al., 2021), scavenge arthropod prey (Hansen and Akre, 1985; Tilles and Wood, 1986), and feed on bird excreta, mammal urine, and carrion (AR pers. obvs). Of the many saccharides present in aphid honeydew, worker ants preferentially consume fructose and sucrose (Renyard et al., 2021) which are widely present also in floral nectar (Blüthgen et al., 2004; Woodring et al., 2004).

The carpenter ants' favorite saccharides also occur in the nectar of fireweed, *Chamaenerion angustifolium* (Anton, et al., 2017), and are likely present in the nectar of thimbleberry, *Rubus parviflorus*, which are two plant species common in forest clearings. The ants may less likely encounter hardhack, *Spiraea douglasii* – which thrives in more riparian habitats – but might still respond to its floral odor when presented with it. Whether carpenter ants are attracted to floral resources has not yet been studied but

pollinivory by ants, including *Camponotus* carpenter ants (Czechowski et al., 2011; Cembrowski et al., 2015), is increasingly observed. Here, we tested the hypotheses that (1) food sources rich in carbohydrates (aphid honeydew, floral nectar) and rich in proteins (bird excreta, house mouse carrion, cow liver infested or not with fly maggots) all prompt long-distance attraction of worker ants, and (2) attraction of worker ants to inflorescences (fireweed, thimbleberry, and hardhack) is mediated by floral semiochemicals that are shared between these plants.

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4.3. Materials and methods

4.3.1. Experimental ants

Nests of *C. modoc* were collected as previously described by Renyard et al. (2019). Briefly, we excised nine nests from forest logs and maintained them in an outdoor undercover area of the Science Research Annex (Burnaby campus, Simon Fraser University), where they experienced natural light and temperature cycles. We housed ant-infested log sections in large plastic bins connected via clear Nalgene™ tubing to glass aquaria provisioned with food (20% sugar water, apples, meal worms, cockroaches) *ad libitum*.

4.3.2. General design of y-tube olfactometer bioassays

Attraction of ants to odor sources was tested in glass Y-tube olfactometers, with odor and control stimuli placed by stratified random assignment (Thompson, 2012) at the orifice of the left or right side arm (Renyard et al., 2019; Figure 4.1). For each bioassay, we disconnected the Nalgene tubing (see above) from an aquarium and allowed a single outbound ant to walk into a glass holding tube inserted into the Nalgene tubing. We then attached the holding tube to the Y-tube olfactometer via a male/female glass joint and drew air at 0.5 L/min through the olfactometer system with a Neptune Dyna vacuum

pump (A.O. Smith, Tipp City, OH, United States). An ant's first choice of side arm was recorded when she crossed a line 6 cm from a side arm's orifice. Ants that did not make a choice within 10 min were considered non-responders and were excluded from statistical analyses. We aimed for 30 replicates per experiment but ran fewer replicates if the test stimulus was obviously not attractive, and we ran more replicates if deemed necessary to avoid statistical error type II. Any ant was bioassayed only once. Following bioassays, counter tops and the rubber stopper connecting the vacuum tubing to the holding tube (Figure 4.1) were cleaned with hexane and ethanol. Y-tubes and holding tubes were cleaned with hot water and soap (Sparkleen, Thermo Fisher Scientific, MA, United States) and dried in an oven for at least 1 h at 100◦C. We ran olfactometer experiments during the summer of 2018, 2019, 2020, and 2021.

4.3.3. Effect of aphid-infested branches on ant attraction (Exp. 1)

To isolate the effects of aphid/honeydew presence as the test variable for ant attraction, we bioassayed aphid-infested vs. uninfested branches. Branches infested, or not, with *Cinara splendens* aphids were cut from a Douglas-fir tree, *Pseudotsuga menziesii*, and inserted into parafilm-covered, water-filled 0.5- dram vials. To ensure that control branches were free of honeydew, they were gently rinsed with water prior to clipping. We then placed one aphid-infested branch and one control branch into separate Ziploc bags (S.C. Johnson and Son, Ltd., Brantford, ON, Canada), cut open one corner of bags to allow air intake, and secured the large opening of bags with a metal hose clamp to the side arms of the Y-tube olfactometer. For each bioassay ant, we used a new aphid-infested branch and a new control branch.

4.3.4. Effect of inflorescences on ant attraction (Exps. 2–4; Table 4.1)

To isolate the inflorescence effect on ant attraction, we bioassayed branches with or without inflorescence, or, a leaf vs. a flower (thimbleberry). Inflorescences and corresponding control branches or leaves of fireweed, thimbleberry, and hardhack were cut from live plants. Each inflorescence and a corresponding control stimulus (see below) were inserted into separate parafilm- covered, water-filled 0.5-dram vials. Hardhack inflorescences consisted of∼130 individual florets, with similar-sized hardhack branches serving as a control stimulus. For thimbleberry, a single flower and a neighboring leaf served as treatment and control stimuli, respectively. For fireweed, a

peduncle with 3–10 flowers and a peduncle with leaves only (control) were tested. Hardhack inflorescences and single thimbleberry flowers, with paired controls, were directedly inserted into side arms of the Y-tube olfactometer. Fireweed inflorescences with paired controls were enclosed in separate Ziploc bags which were then attached to the side arms of Y-tube olfactometers. For each bioassay ant, we tested new plant material.

4.3.5. Effect of fresh and insect-infested carrion on ant attraction (Exps. 5–7; Table 4.1)

CO2-euthanized female house mice, *Mus musculus*, and beef liver from recently slaughtered cows were tested as fresh carrion sources in parallel Y-tube olfactometer experiments. House mice were salvaged from an unrelated experiment which required removal of their uterus followed by freezing. Bisected mice (Exp. 5) and mass-matched beef liver pieces (each ~6 g) (Exp. 6) were wrapped in cheese cloth and frozen until use. Prior to testing in bioassays, samples were thawed in warm water and then placed in a glass tube (60 mm × 19 mm inner diam.) kept on ice. For each replicate, paired tubes containing either the test sample wrapped in cheese cloth or cheese cloth only (control) were inserted into the side arms of the Y-tube olfactometer.

To obtain aged, maggot-infested carrion (Exp. 7), a 354-mL paper cup (Solo Cup Company, IL, United States) containing a piece of beef liver (2.5 cm × 7.5 cm) was placed into a cage (61 cm × 61 cm × 61 cm; BioQuip®, Compton, CA, United States) with 500 male and female blow flies, *Phormia regina*, allowing females 4 h to oviposit on the liver. After the 4-h period, the cup was removed, another piece of liver (2.5 cm × 7.5 cm) was added, and the cup was covered with mesh and paper towel and kept in a veiled bin (70.5 cm × 36 cm × 11.5 cm). First-instar maggots were transferred to a glass jar (11 cm× 16.5 cm× 16.5 cm) containing both a chunk of liver (250 g) and wood shavings, and were allowed to develop to 3rd instars which were tested in bioassays. For these bioassays, three 3rd instar maggots, along with the same liver (1-g aliquots) in which they had developed, were enclosed in an 8-layer cheesecloth pouch. Maggot-infested liver pouches and empty control pouches were placed into separate glass tubes (60 mm × 19 mm) and kept on ice in separate coolers prior to bioassays. For each replicate, paired tubes containing either a maggot-infested liver pouch or a blank control pouch were inserted into the side arm of the Y-tube olfactometer. To test the effects of

dead mice, fresh and maggot-infested liver, or bird droppings (see below) on ant attraction, we considered air the only appropriate control stimulus as any other 'control' odor may have altered the ants' responses. For each bioassay ant, we tested new stimuli.

4.3.6. Effect of bird excreta on ant attraction (Exp. 8; Table 4.1)

Fresh excreta of Ruffs, *Calidris pugnax* – maintained in SFU's Animal Care facility for another project – were collected with a scoopula from the ground and placed in a Petri dish. Excreta were homogenized and used within 24 h of collection. On each bioassay day, we placed aliquots of bird excreta (0.5–0.6 g) into glass tubes (60 mm × 19 mm) and covered openings with metal mesh. Paired tubes with, or without (control), bird excreta were placed in separate Styrofoam coolers containing ice. For each replicate, we inserted a glass tube containing bird excreta into one side arm of a Y-tube olfactometer and an empty control tube in the corresponding control side arm. For each bioassay ant, we tested new stimuli.

4.3.7. Collection of headspace volatiles of attractive food sources

As fireweed, thimbleberry, and bird excreta attracted ants (see Section "Results"), we collected their headspace volatiles for analyses. Driven by a vacuum pump (Neptune Dyna; A.O. Smith, Tipp City, OH, United States), air was drawn at 1 L min−1 for 16–24 h through activated charcoal, through a glass chamber (41 cm × 17.5 cm diameter) containing the odor source, and finally through a glass tube (14.0 cm × 0.5 cm) filled with Porapak Q adsorbent (200 mg) (Figure 4.1). Volatiles were desorbed from Porapak Q by flushing it with 2 mL of ether/pentane (1/1). For fireweed headspace volatile collections, 162 flowers from 20 plants were aerated for 19 h, yielding a total of 3,078 flower- hour equivalents (FHEs) of headspace volatile extract. Aliquots of this extract were tested in behavioral bioassays (Exps. 9– 10; below), and extract analyses informed the preparation of a synthetic blend tested in experiment 14.

Thimbleberry headspace flower volatiles were collected on two dates: (1) in 2019 (when most thimbleberry shrubs had already finished blooming), five flowers were aerated for 16 h, yielding a total of 80 FHEs of headspace volatile extract; (2) in 2020, 31 flowers were aerated for 24 h, yielding a total of 744 FHEs of headspace volatile extract.

Analysis of the 2019- extract informed the preparation of the synthetic blend tested in experiment 15 (see below).

For headspace volatile collections of bird excreta, 16.6 g of excreta from Ruffs were aerated for 24 h, yielding a total of 397.2 gram-hour equivalents (GHEs) of headspace volatile extract.

4.3.8. Analyses of headspace volatile extracts by gas chromatography-mass spectrometry

Extracts of fireweed, thimbleberry, and bird excreta were concentrated under a nitrogen stream to 200, 130, and 120 µL, respectively, and 2-µL aliquots of each concentrate were analyzed by gas chromatography-mass spectrometry. GC-MS analyses deployed an Agilent GC-MS (Agilent 7890B GC coupled to a 5977A Series MSD; Agilent Technologies Inc., Santa Clara, CA, United States) fitted with a DB-5 column (30 m × 0.25 mm ID.; Agilent Technologies, see above), using helium as the carrier gas (35 cm s−1) and the following temperature program: 50◦C (held for 5 min), 10◦C per min to 280◦C (held for 10 min). Samples were analyzed in split mode (5:1 ratio), with the injector port set to 250◦C, the transfer line to 280◦C, the MS Quadrupole to 150◦C, and the MS source to 230◦C. Compounds were identified by comparing their retention indices (Van den Dool and Kratz, 1963) and mass spectra with those of authentic standards.

Sources of authentic chemical standards

The sources and purities of authentic chemical standards are listed in Table 4.2.

4.3.9. Attraction of ants to headspace volatile extracts of attractive food sources

General bioassay Design

Headspace volatile extracts and synthetic volatile blends were tested in Y-tube olfactometers at doses equivalent to volatiles released from natural test stimuli during 10-min bioassays (see Exps. 3, 4, 8). We also tested synthetic blends at a 10-fold higher dose to account for different release dynamics between synthetic and natural sources. Synthetic blends were formulated in pentane/ether (1/1) and 10-µL aliquots were applied

to a piece (1 cm × 1 cm) of cotton dental wick (Richmond Dental & Medical, Charlotte, NC, United States) at the orifice of an olfactometer side arm. In each bioassay, the piece of cotton wick in the control side arm received the corresponding volume (10 µL) of pentane/ether.

4.3.10. Specific experiments

Effect of fireweed extract on ant attraction (Exps. 9, 10; Table 4.1)

Drawing on results that fireweed inflorescences with 3–10 flowers each (median: 7 flowers) attracted ants in 10-min (0.167-h) bioassays (see Section “Results” in Exp. 3), we tested headspace volatile extract in experiment 9 at 1.16 FHEs per replicate (7 flowers × 0.167 h = 1.16 FHEs). Predicting rapid (rather than sustained) release of synthetic volatiles from cotton wicks, we also tested a 10-fold higher dose (11.6 FHEs; Exp. 10).

Effect of thimbleberry extract on ant attraction (Exp. 11; Table 4.1)

Drawing on results that one thimbleberry flower was sufficient to attract ants in 10-min bioassays (see Section “Results” in Exp. 4), we were inclined to test headspace volatile extract in experiment 11 at 0.167 FHEs per replicate (1 flower × 0.167 h = 0.167 FHE). However, as fireweed extract was effective only at a 10× higher dose, we instead tested 1.67 FHEs (Exp. 11).

Effect of bird excreta on ant attraction (Exps. 12–13; Table 4.1)

Drawing on results that 0.5 g of bird excreta attracted ants in 10-min bioassays (see Section “Results” in Exp. 8), we tested headspace volatile extract in experiment 12 at 0.084 GHEs per replicate (0.5 g of bird excreta × 0.167 h = 0.084 GHE). Considering that 0.5 g of bird excreta were very attractive to ants (Exp. 8), we tested headspace volatile extract at both a lower dose (0.084 GHE; Exp. 12) and a 10-fold higher dose (0.84 GHE; Exp. 13).

Effect of synthetic volatile blends of fireweed, thimbleberry and bird excreta on ant attraction (Exps. 14–16; Table 4.1)

Drawing on results of experiments 9–13, synthetic volatile blends of fireweed inflorescences, thimbleberry flowers, and bird excreta were tested at a 10× dose,

comprising 11.6 FHEs, 1.67 FHEs, and 0.84 GHEs, respectively (Table 4.1). Synthetic blends (SBs) were formulated in pentane/ether (1/1), and 10-µL aliquots of formulations, or of pentane/ether control stimuli, were applied to a cotton wick at the orifice of olfactometer side arms.

Effect of volatiles shared between fireweed and thimbleberry on ant attraction (Exp. 17; Table 4.1)

As ants were attracted to synthetic volatile blends of fireweed inflorescences and thimbleberry flowers (see Section "Results" of Exps. 14 and 15), we proceeded to test volatiles [(*E*)-β - caryophyllene, α-humulene] that are shared between these plants. We presented these two compounds at the same 10× dose as tested in experiments 15 (Table 4.1).

4.3.11. Statistical analysis

Data (Renyard et al., 2022) were analyzed and graphics prepared using R (V4.0.3; R Core Team, 2020), RStudio (Version 1.4.1103) and Inkscape (Version 1.0.2). Data from all two-choice Y-tube olfactometer experiments were analyzed with a χ2 test against a theoretical 50:50 distribution, under the null hypothesis that treatment stimuli have no effects on the ants' choices.

4.4. Results

4.4.1. Effects of aphid-infested branches, floral volatiles, carrion and bird excreta on ant attraction (Exps. 1–8)

In two-choice Y-tube olfactometer experiments (Figure 4.2), ants preferred fireweed inflorescences to fireweed leaves ($\chi^2 = 4.8$, df = 1, n = 30, p = 0.0285), thimbleberry flowers to thimbleberry leaves ($\chi^2 = 4.8286$, df = 1, n = 35, p = 0.0280), and bird excreta to clean air ($\chi^2 = 4.84$, df = 1, n = 25, p = 0.0278). Ants showed no preference when offered choices between (i) aphid- infested branches and control branches, (ii) hardhack flowers and hardhack leaves, (iii) mouse carrion and clean air, (iii) cow liver and clean air, and (iv) maggot-infested cow liver and clean air (all p > 0.05).

4.4.2. Identification of compounds in attractive headspace volatile extracts

Headspace volatile extracts of the three sources (fireweed, thimbleberry, and bird excreta) that were attractive to ants proved complex. Thimbleberry headspace volatiles included three hydrocarbons, two alcohols, one ketone, and one methyl ester (Figure 4.3). Fireweed headspace volatile included six sesquiterpenes, five esters, three alcohols, four ketones, three aldehydes, one triene hydrocarbon, and one methoxy alcohol (Figure 4.3). Bird excreta headspace volatiles consisted of seven ketones, five alkanes, four acids, three alcohols, three aldehydes, three pyrazines, two sulfides, two nitriles, two esters, one keto- alcohol, one acetate, one monoterpene, one isothiocyanate, one methoxy alcohol, one benzene pyrrole, and one thionitrile (Figure 4.4). (*E*)-β-Caryophyllene and α-humulene were shared between thimbleberry and fireweed.

4.4.3. Effect of headspace volatile extracts of fireweed, thimbleberry, and bird excreta on ant attraction (Exps. 9–13)

When ants in Y-tube olfactometer experiments were offered choices between solvent control stimuli and (i, ii) headspace volatile extracts of fireweed [1× dose (Exp. 9); 10× dose (Exp. 10)], (iii) thimbleberry [10× dose (Exp. 11)], and (iv, v) bird excreta [1× dose (Exp. 12); 10× dose (Exp. 13)], they favored 10× doses of fireweed (Exp. 10: χ^2 = 3.9032, df = 1, n = 31, p = 0.0482), thimbleberry (Exp. 11: χ^2 = 4.8, df = 1, n = 30, p = 0.0285), and bird excreta (Exp. 13: χ^2 = 5.1429, df= 1, n = 28, p = 0.0233; Figure 4.5). In contrast, they did not prefer 1× doses of fireweed (Exp. 9) and bird excreta (Exp. 12) to solvent controls (each p > 0.05).

4.4.4. Effect of synthetic volatile blends of fireweed, thimbleberry and bird excreta on ant attraction (Exps. 14–16)

When ants in Y-tube olfactometer experiments were offered choices between solvent control stimuli and synthetic volatile blends (10× dose) of fireweed, thimbleberry, and bird excreta, they preferred blends of fireweed (Exp. 14: 5.8276, df = 1, n = 29, p = 0.0158) and thimbleberry (Exp. 15: χ^2 = 4.8, df = 1, n = 30, p = 0.0285), but not of bird excreta (Exp. 16: χ^2 = 0.030303, df = 1, n = 33, p = 0.86) to solvent controls (Figure 4.6).

4.4.5. Effect of volatiles shared between fireweed and thimbleberry on ant attraction (Exp. 17)

When ants in Y-tube olfactometer experiment 17 were offered a choice between a synthetic blend of floral odorants shared between thimbleberry and fireweed [(*E*)-β-caryophyllene, α- humulene] and a solvent control stimulus, they showed no preference for either test stimulus (χ^2 = 2.7931, df = 1, n = 29, p = 0.095; Figure 4.7).

4.5. Discussion

Ant colonies have vast and diverse nutritional needs including carbohydrates and proteins (Porter, 1989; Evans and Pierce, 1995; Feldhaar et al., 2007; Blüthgen and Feldhaar, 2010; Mankowski and Morrell, 2014), but forager ants have limited mobility to meet these needs. Foragers face the challenge of not only finding enough nutrients for themselves but also for all of their nestmates including the developing brood (Csata and Dussutour, 2019). Foragers would likely accrue significant energy savings for themselves and for their entire colony if they were able to sense olfactory cues from both carbohydrate and protein sources, and to engage in long-distance orientation toward them. If forager ants had reduced olfactory acuity, like other flightless insects (Neupert et al., 2020), they might not be able to recognize the specific odor profiles of multiple food sources but might still be able to locate them by responding to key odorants shared between food sources. Working with Western carpenter ants as a model species, we show that foragers are capable of long-distance orientation toward both carbohydrate and protein food sources. Foragers were attracted to two of four carbohydrate sources (thimbleberry and fireweed) and to one of four protein sources (bird excreta) that we tested in bioassays. However, a blend of floral odorants shared between thimbleberry and fireweed was not attractive to ants, indicating that select floral odorants, while common among plants, are not attractive to ants when presented outside typical floral odor context. As ants were selective in their responses to carbohydrate and protein food sources, we conclude that they can discern between specific food odor profiles, and that they seem to have good, rather than poor, olfactory acuity.

All four sources of carbohydrates (aphid-infested conifer branches, blooming fireweed, thimbleberry, and hardhack) that we tested in our study would have provided nutritional value to foraging ants. Thus, it is surprising that aphid-infested branches and

hardhack did not attract ants. Honeydew, in particular, is consumed by many species of ants – including *C. modoc* (Tilles and Wood, 1986; Yamamoto and Del-Claro, 2008; Ness et al., 2010; Renyard et al., 2021) – and may constitute a large proportion of an ant's diet (Domisch et al., 2009; Pekas et al., 2011). That worker ants of *L. niger* and *S. invicta* were attracted to honeydew (Zhou et al., 2012; Fischer et al., 2015), but *C. modoc* workers were not (Figure 4.2), has at least three plausible explanations: (1) there simply may not have been sufficient honeydew accumulation on the aphid- infested branches; (2) the aphid honeydew, which at the time of excretion is odorless (A.R.; pers. observ.), was not yet extensively colonized by exogenous microbes whose volatile metabolites attract natural enemies to aphid colonies (Leroy et al., 2011), and also accounted for attraction of *L. niger* workers (Fischer et al., 2015); and (3) *Cinara* aphids colonize tall conifer trees and their honeydew odors, or alarm pheromone signals, may not consistently reach ground-dwelling carpenter ants, providing little opportunity for ants to associate *Cinara* honeydew odor or aphid pheromones with the presence of aphids and carbohydrate rewards (Verheggen et al., 2012).

Insufficient overlap between habitats colonized by carpenter ants and hardhack may also explain the non-attractiveness of hardhack flowers. Hardhack thrives in open riparian habitats (Pojar et al., 1994) and is less common in areas frequented by carpenter ants (A.R.; pers. observ.), whereas fireweed and thimbleberry are common plant community members of the forest ecosystem (Pojar et al., 1994) inhabited by carpenter ants. Alternatively, the nectar or pollen rewards of hardhack are not sufficiently appealing to, or accessible by, carpenter ants.

Animal-derived nitrogenous sources such as bird excreta, vertebrate urine, and carrion are ephemeral resources. If foraging ants were to rely on chance encounters of these resources, they might not be able to meet the protein requirements of their colony's egg-laying queen and developing brood. Expectedly then, worker ants were attracted to fresh bird excreta (Figure 4.2). While ants are known to forage on bird excreta (Kaspari, 1993; Jaffe et al., 2001; Sainz-Borgo, 2015), their olfactory attraction to bird excreta has not previously been reported. Bird excreta are nutritionally valuable to carpenter ants not only as a protein source, but also as a source of uric acid and urea. The ants' obligate endosymbiont *Blochmannia spp*. enzymatically breaks down uric acid and urea, and converts urea to both essential and non-essential amino acids (Sauer et al., 2000; Feldhaar et al., 2007). This metabolic capability improves the nutritional intake

of the host ants, enables them to persist on otherwise nutrient-deficient diets, and allows them to occupy nutritional niches off-limits to ant community members lacking these endosymbionts (Davidson et al., 2003; Feldhaar et al., 2007; Russell et al., 2009; Hu et al., 2018). The ants' endosymbiotic ability to process urea as an amino acid precursor may also explain their lack of attraction to other protein sources, such as mouse carrion (Figure 4.2). Several species of carpenter ants selectively feed on urea (Shetty, 1982; Feldhaar et al., 2007; Menzel et al., 2012), and worker ants of *Camponotus terebrans* even sift through sand containing urea to acquire it (Petit et al., 2020).

Although our synthetic blend of bird excreta odorants was very complex, it still failed to attract ants in Y-tube olfactometers, indicating that essential constituents were still missing from the blend. These constituents could have been too polar to properly chromatograph [e.g., (bi)acids] or too low in abundance to be detectable in GC-MS analyses.

Over 154 species of ants, including *C. modoc* carpenter ants (Shean et al., 1993), have been found on or near carrion (Eubanks et al., 2019) but – surprisingly – neither mouse carrion nor cow liver at various stages of decay attracted carpenter ants in our study. The odor profile of decaying carrion dynamically changes in relation to the stage of decay (Dekeirsschieter et al., 2009), and each stage attracts a different guild of scavengers. For example, very fresh carrion attracts blow flies (Brodie et al., 2016), whereas the dry (bone and hair only) stage is attractive to clothes moths (Takács et al., 2001). The carrion stage that is preferentially sought by scavenging ants has not yet been investigated, and we may have presented a suboptimal stage in our bioassays. Alternatively, scavenging ants may prefer invertebrate to vertebrate protein, and vertebrate carrion protein is a suboptimal food source. This explanation is supported by findings that laboratory colonies of *S. invicta* and *Solenopsis geminata* had greater brood production and growth when provisioned with insect protein instead of liver protein (Gavilanez-Slone and Porter, 2013; Porter et al., 2015; Lin et al., 2022). It follows that ant assemblies on vertebrate carrion may be motivated primarily by prospective encounters with insect prey, such as fly maggots developing in carrion (Lin et al., 2022). In our study, the liver odor may have masked the (faint) fly maggot odor.

Our prediction that foraging ants are reliant upon a simple olfactory search 'image' for nectar odor cues was not supported by the data. The prediction was inspired

by previous reports that flightless insects have poor olfactory acuity (Neupert et al., 2020). With a simple search image, 'featuring' only those floral constituents that are shared between plants, foraging ants would be able to locate and exploit multiple and diverse nectar sources. This concept seemed particularly appealing because more than 50% of flowering plant families have floral bouquets with overlapping constituents, including α-pinene, benzaldehyde, linalool, and *E*-β-caryophyllene (Knudsen et al., 2006). *E*-β-Caryophyllene and α-humulene are shared between fireweed and thimbleberry but this 2-component blend was not attractive to ants. This finding, coupled with (i) reports that ants have hundreds of olfactory receptors (Saad et al., 2018), and (ii) our data showing that foraging carpenter ants were attracted to complete floral odor bouquets of fireweed and thimbleberry but not of hardhack, support the conclusion that carpenter ants recognize specific floral odor blends and discern between them, and thus have good, rather than poor, olfactory acuity.

Western carpenter ants had no prior contact or experience with the carbohydrate and protein sources we tested, indicating innate responses. However, ants in general can learn to associate odors with food rewards (Dupuy et al., 2006; Provecho and Josens, 2009; Nelson et al., 2019; Oberhauser et al., 2019), and thus may be able to opportunistically adjust their foraging activities in accordance with the resources that are currently available in their habitat and that they have learned about. The ants' disposition to respond to olfactory cues is likely affected by both resource-specific factors such as an optimally attractive stage (see above) and intrinsic 'ant' factors such as caste, stage, or hunger (Morgan et al., 2006; Seid and Traniello, 2006; Muscedere et al., 2012; Gadenne et al., 2016). The disposition to respond to olfactory cues is further modulated by shifting barometric pressure (Pellegrino et al., 2013) which could explain the variable number of non-responding ants in our bioassays. Spatio-temporal overlap of food and ant presence, and nutritional value of food, are obvious requisites for odor-mediated foraging responses by ants.

4.5.1. Conclusion

Foragers of Western carpenter ants are attracted to food sources rich in carbohydrates and proteins. The foragers' ability to sense and orient toward sources of these two macronutrients greatly improves their foraging efficiency. Foragers are not reliant on chance encounters of these resources but can detect them from a distance

and move upwind toward them. This ability likely translates into significant energy savings for the entire colony. A complex, rather than simple, olfactory search image seems to guide the foraging activities of ants. They discriminated between odor profiles of three flowering plants, selecting only two (fireweed and thimbleberry), and four protein sources, selecting only one (bird excreta). Moreover, the simple blend of only those two floral odorants shared between fireweed and thimbleberry had no 'ant appeal.' All these data support the conclusion that carpenter ants have significant olfactory acuity. With a keen sense of smell, flightless forager ants can efficiently locate valuable nutrient sources and meet the vast and diverse nutritional needs of all their worker nestmates, queen, and developing brood.

4.6. Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: doi: 10.17632/7kj4s38rvn.

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4.8. References

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4.9. Tables

Table 4.1. Stimuli tested with *Camponotus modoc* ants in experiments 1–17

Exp. #	Stimuli tested	No. ants responding (not-responding)
(H1) Food sources rich in carbohydrates (aphid honeydew, floral nectar) and rich in proteins (bird excreta, house mouse carrion, cow liver infested or not with fly maggots) all prompt long-distance attraction of worker ants		
1	Aphid-infested branch *vs.* uninfested branch	20 (0)
2	Hardhack inflorescence *vs.* hardhack leaf	20 (7)
3	Fireweed inflorescence *vs.* fireweed leaves	30 (10)
4	Thimbleberry flower *vs.* thimbleberry leaf	35 (1)
5	House mouse carrion *vs.* blank control	30 (5)
6	Cow liver *vs.* blank control	30 (3)
7	Maggot-infested cow liver *vs.* blank control	30 (2)
8	Bird excreta *vs.* blank control	25 (4)
9	Fireweed HVE[a] (1×) *vs.* solvent control	30 (3)
10	Fireweed HVE (10×) *vs.* solvent control	31 (2)
11	Thimbleberry HVE (10×) *vs.* solvent control	30 (1)
12	Bird excreta HVE (1×) *vs.* solvent control	29 (1)
13	Bird excreta HVE (10×) *vs.* solvent control	28 (2)
14	Fireweed SB[b] (10×) *vs.* solvent control	29 (3)
15	Thimbleberry SB[c] (10×) *vs.* solvent control	30 (0)
16	Bird excreta SB[d] (10×) *vs.* solvent control	33 (3)
(H2) Attraction of worker ants to inflorescences (fireweed, thimbleberry, hardhack) is mediated by floral semiochemicals that are shared between these plants.		
17	Odorants shared between fireweed & thimbleberry (10×) *vs.* solvent control	29 (1)

[a] Headspace volatile extracts (HVE) and synthetic blends (SB) were tested at doses equivalent (1×) to volatiles released from natural sources (Exps. 3,4,8) or 10-fold higher (10×).

[b] Fireweed SB (10×): ethyl butyrate (2.5 ng), ethyl-2-methyl butyrate (2.5 ng), (Z)-3-hexenol (6 ng), 2-heptanone (4.5 ng), (*Z*)-5-hepten-2-one (2 ng), benzaldehyde (0.5 ng), sulcatone (1 ng), phenylacetaldehyde (4 ng), 2-nonanone (2 ng), linalool (2 ng), nonanal (2 ng), phenylethylalcohol (1.3 ng), (*E*)-4,7-dimethyl-1,3,7-nonatriene (1.3 ng), (*Z*)-3-hexenylbutyrate (6.5 ng), methylsalicylate (1 ng), (*Z*)-3-hexenyl-2-methyl-butyrate (5 ng), 4-Allyl-2-methoxyphenol (5 ng), β-elemene (10 ng), (*E*)-β-caryophyllene (11 ng), (*E*)-β-farnesene (1 ng), α-humulene (3.9 ng), germacrene D (5 ng), (*E,E*)-α-farnesene (3 ng)

[c] Thimbleberry SB (10×) [b]: α–pinene (209.8 ng), methylbenzoate (6.3 ng), (–)-*cis*-verbenol (4.2 ng), (–)-*trans*-verbenol (4.2 ng), (–)-verbenone (25.1 ng), (*E*)-β-caryophyllene (137.8 ng), α-humulene (54.3 ng)

[d] Bird excreta SB (10×): isoamyl alcohol (0.3 ng), dimethyl disulphide (5.3 ng), 2,4-pentadiene-nitrile (0.3 ng), isobutryic acid (0.5 ng), 3-hexanone (1.3 ng), butyric acid (0.5 ng), 2-hexanone (2.5 ng), octane (1.3 ng), 2-hydroxy-3-pentanone (0.5 ng), ethyl-2-methylbutyrate (0.1 ng), 3-methylbutyric acid (0.3 ng), ethyl-3-methylbutyrate (0.1 ng), 2-methylbutyric acid (0.3 ng), isoamyl acetate (0.3 ng), 2-heptanone (0.8 ng), nonane (0.5 ng), 2,5-dimethylpyrazine (0.5 ng), 2,3-dimethylpyrazine (0.5 ng), α-pinene (0.5 ng), benzaldehyde (1.3 ng), dimethyl trisulphide (0.5 ng), phenol (0.5 ng), 4-isothiocyanate-1-butene (2.5 ng), 1-octen-3-ol (12.6 ng), 3-octanone (15.1 ng), 2-octanone (0.5 ng), tetramethylpyrazine (2 ng), guaiacol (2 ng), nonanal (3.5 ng), 5-methylthiopentanenitrile (3.3 ng), decanal (1.3 ng), 2-undecanone (0.5 ng), indole (0.8 ng), tridecane (0.5 ng), 5-methylthiohexanenitrile (1.5 ng), geranyl-acetone (1.3 ng), pentadecane (0.3 ng), pristane (3 ng)

Table 4.2. Chemical numbers (No.; see Figs. 4.3, 4.4), suppliers, and purities of chemicals tested in Y-tube olfactometer bioassays.

#	Chemical	Supplier	% Purity (%)	#	Chemical	Supplier	% Purity (%)
1	(–)-α-Pinene	SA[a]	98	32	Isobutyric acid	SA	99
2	Methylbenzoate	SA	98	33	3-Hexanone	SA	98
3	(–)-*cis*-Verbenol	FI[b]	>95	34	Butyric acid	SA	99
4	(–)-*trans*-Verbenol	PT[c]	95	35	2-Hexanone	SA	98
5	(–)-Verbenone	PT	>95	36	Octane	SA	98
6	(*E*)-β-caryophyllene	SA	99	37	2-Hydroxy-3-pentanone	GL[l]	90
7	α-Humulene	SA	99	38	3-Methylbutyric acid	SA	99
8	Ethyl butyrate	GL[d]	95	39	Ethyl 3-methylbutyrate	GL[d]	95
9	Ethyl 2-methylbutyrate	GL[d]	95	40	2-Methylbutyric acid	SA	98
10	(*Z*)-3-Hexenol	SA	98	41	Isoamyl acetate	GL[m]	98
11	2-Heptanone	SA	95	42	Nonane	SA	98
12	(*Z*)-5-Hepten-2-one	GL[e]	95	43	2,5-Dimethylpyrazine	SA	98
13	Benzaldehyde	SA	95	44	2,3-Dimethylpyrazine	SA	99
14	6-Methyl-5-hepten-2-one	SA	99	45	Dimethyl trisulfide	SA	98
15	Phenylacetaldehyde	SA	>90	46	Phenol	SA	99
16	2-Nonanone	SA	95	47	4-Isothiocyanate-1-butene	GL[n]	65
17	(–)-Linalool	FI	97	48	1-Octen-3-ol	SA	98
18	Nonanal	SA	95	49	3-Octanone	SA	98
19	Phenylethyl alcohol	FI	>99	50	2-Octanone	SA	98
20	(*E*)-4,7-Dimethyl-1,3,7-nonatriene	GL[f]	98	51	Tetramethylpyrazine	SA	98
21	(*Z*)-3-Hexenyl butyrate	GL[d]	95	52	Guaiacol	FI	>98
22	Methylsalicylate	SA	99	53	5-Methylthiopentanenitrile	GL[o]	99

23	(*Z*)-3-Hexenyl 2-methyl-butyrate	GL[d]	95	54	Decanal	SA	99
24	4-Allyl-2-methoxyphenol	SA	95	55	2-Undecanone	SA	98
25	β-Elemene	GL[g]	99	56	Indole	SA	99
26	(*E*)-β-Farnesene	Be[h]	95	57	Tridecane	SA	99
27	Germacrene D	GL[i]	93	58	5-Methylthiohexanenitrile	GL[p]	99
28	(*E,E*)-α-Farnesene	PT[c]	65	59	Geranylacetone	SA	96
29	Isoamyl alcohol	Fi[j]	95	60	Pentadecane	SA	99
30	Dimethyl disulfide	SA	99	61	Pristane	SA	98
31	2,4-Pentadiene nitrile	GL[k]	95				

[a] Sigma-Aldrich (St. Louis, MO 63103, USA).

[b] Fluka Chemie (Buchs, 9471, CH).

[c] Phero Tech Inc. (Delta, BC V4G 1E9, CA) (out of business).

[d] Synthesized in the Gries-lab according to Nieses and Steglich (1978), and purified to >95% by flash chromatography using 10-20% ether in pentane.

[e] Available in the Gries-lab from a previous project

[f] Synthesized in the Gries-lab according to Maurer et al. (1986).

[g] Purified by HPLC from Juniper berry oil (product # 371) supplied by Liberty Natural Products (Oregon City, OR 97045, USA).

[h] Bedoukian Research Inc. (Danbury, CT 06810, USA).

[i] Purchased from Treatt PLC (Lakeland, FL 33805, USA) (40% technical grade) and purified to 93% according to a procedure in Peach et al. (2019).

[j] Fischer Scientific (Janssen Pharmaceuticalaan 3a, 2440 Geel, BE).

[k] Synthesized in the Gries-lab according to Clary and Back (2007)

[l] Synthesized in the Gries-lab according to Moriarty and Hou (1984).

[m] Synthesized in the Gries-lab by converting isoamyl alcohol to its corresponding acetate using acetic anhydride.

[n] Synthesized in the Gries-lab according to Terada et al. (2015).

[o] Synthesized in the Gries-lab according to Moon et al. (2010).

[p] Synthesized in the Gries-lab according to Teders et al. (2018).

4.10. Figures

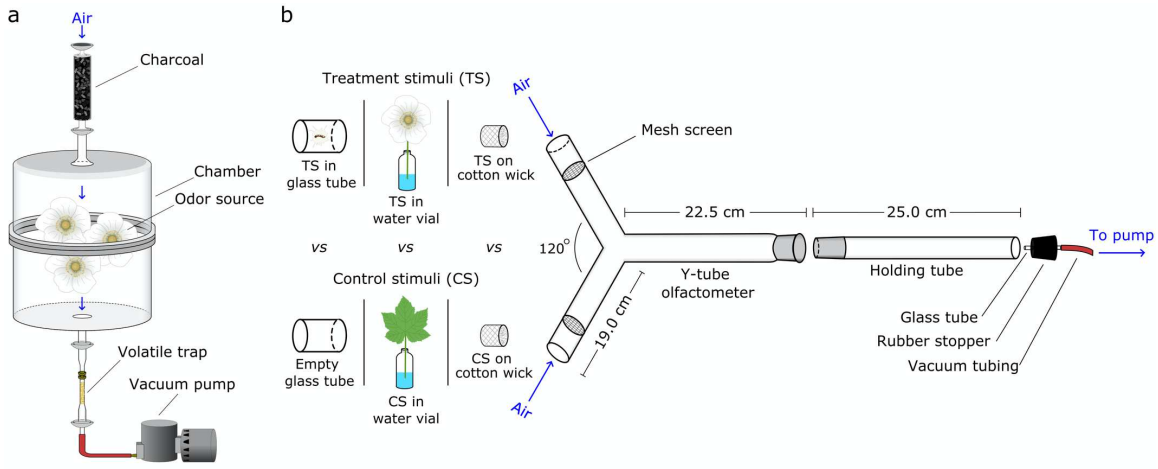

Figure 4.1. **Graphical illustrations of experimental designs. (A) Set-up for collecting headspace volatiles from natural sources; air was drawn through activated charcoal, a glass chamber containing a natural odor source, and a volatile trap (the adsorbent Porapak in a glass tube). (B) Y-tube olfactometer bioassay station for testing attraction of ants to test stimuli; for each replicate, test stimuli were (i) placed in small plastic bags secured to the side arms of the Y-tube, or (ii) inserted directly into the side arms. Then, the holding tube housing a single bioassay ant was connected to both a vacuum pump and the Y-tube, allowing the ant to walk upwind toward test stimuli. Test stimuli consisted of natural odor sources, headspace volatile extracts (HVEs), and synthetic blends (SBs) of candidate semiochemicals. Aliquots of HVEs, SBs and corresponding solvent control stimuli were pipetted onto pieces of cotton wick placed at the orifice of side arms (see Table 4.1 for details).**

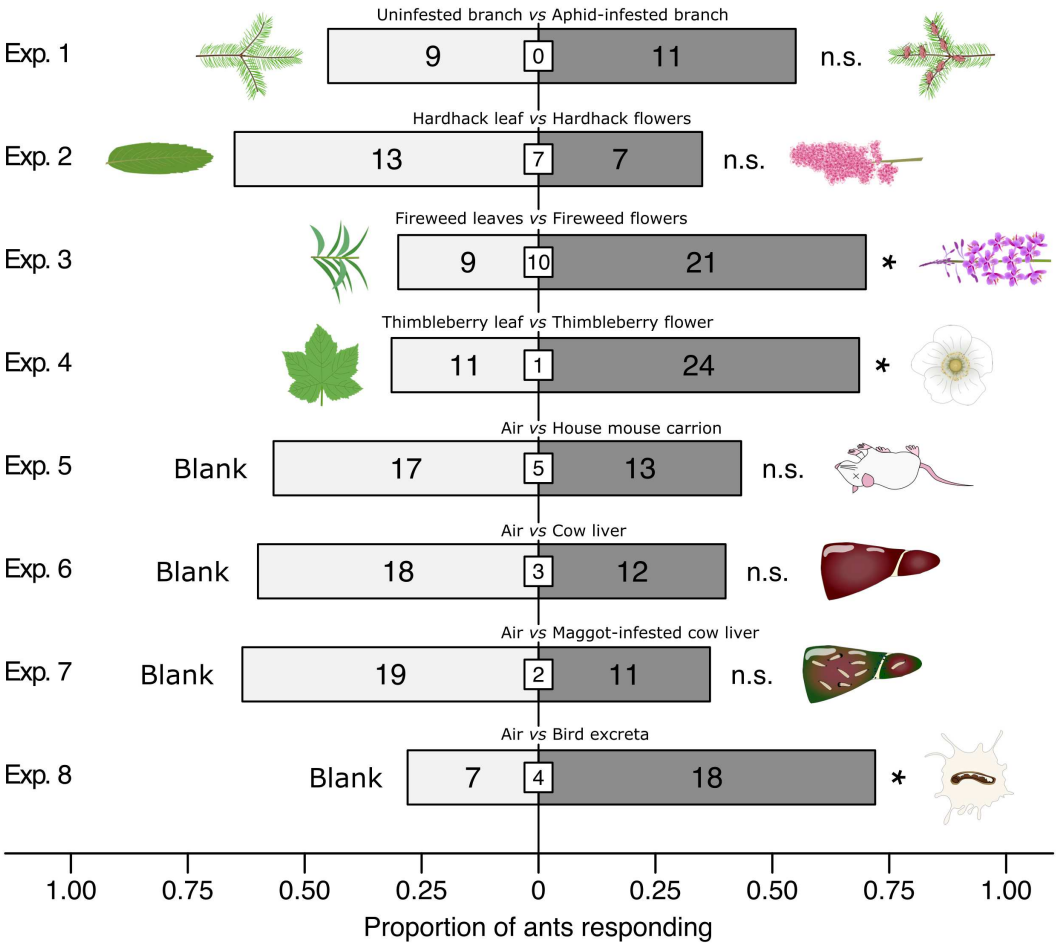

Figure 4.2. **Effect of test stimuli on responses of *Camponotus modoc* worker ants in Y-tube olfactometer (Figure 4.1) experiments 1–8, each with 20–35 responding ants. Treatment and control stimuli are indicated above bars and illustrated next to bars. Numbers within bars indicate the number of ants responding to test stimuli and numbers in white inset boxes indicate the number of non-responding ants. An asterisk denotes a significant preference for a test stimulus (Pearson's χ^2 tests: $p < 0.05$; n. s., not significant)**

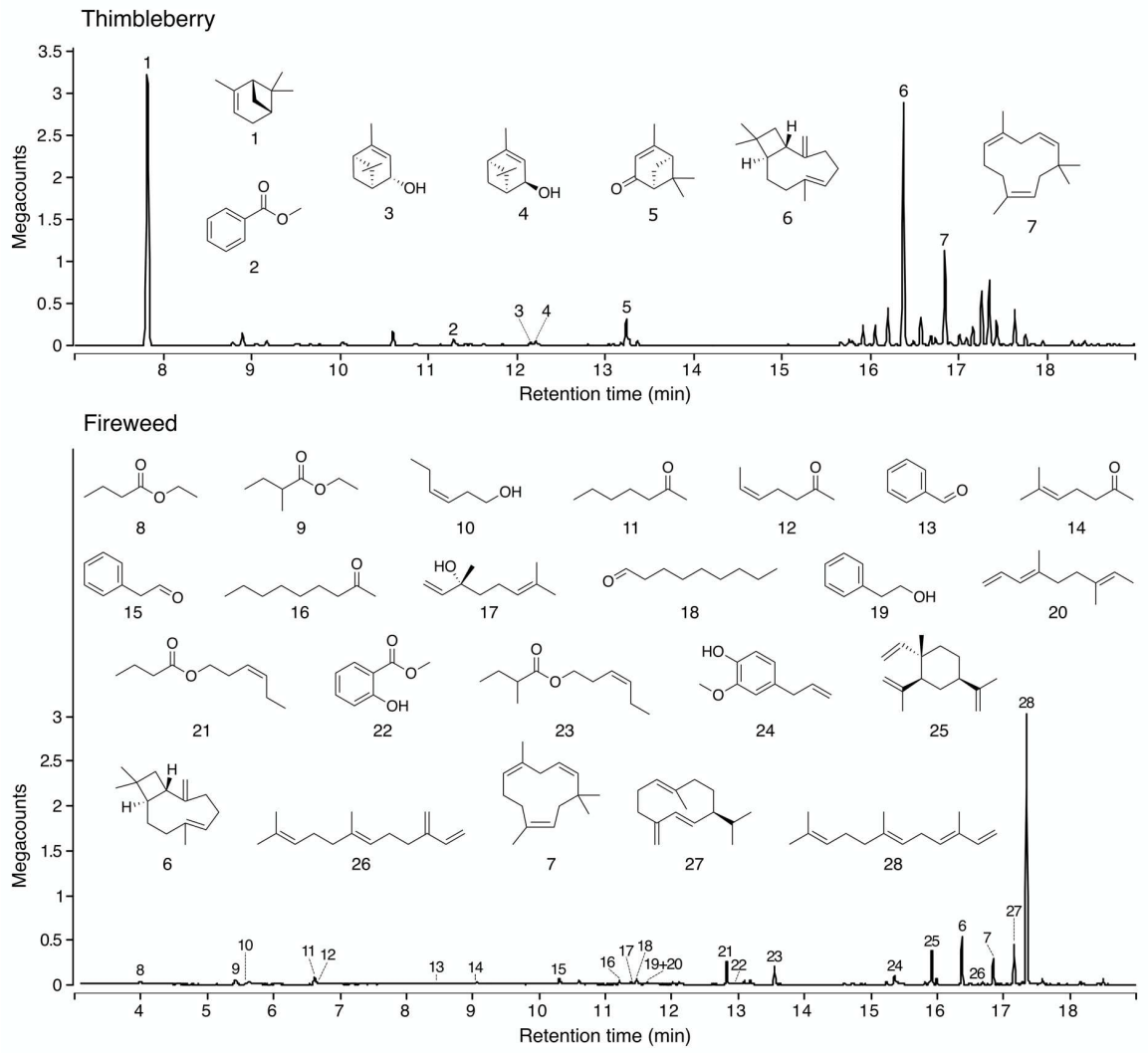

Figure 4.3. Total ion chromatograms of headspace volatile extracts (HVEs) of thimbleberry (Top) and fireweed (Bottom). Headspace volatiles were adsorbed on Porapak Q (Figure 4.1) and desorbed with pentane/ether (1/1). HVEs were analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 7890B GC coupled to a 5977A Series MSD). Names of compounds are listed in Table 4.2.

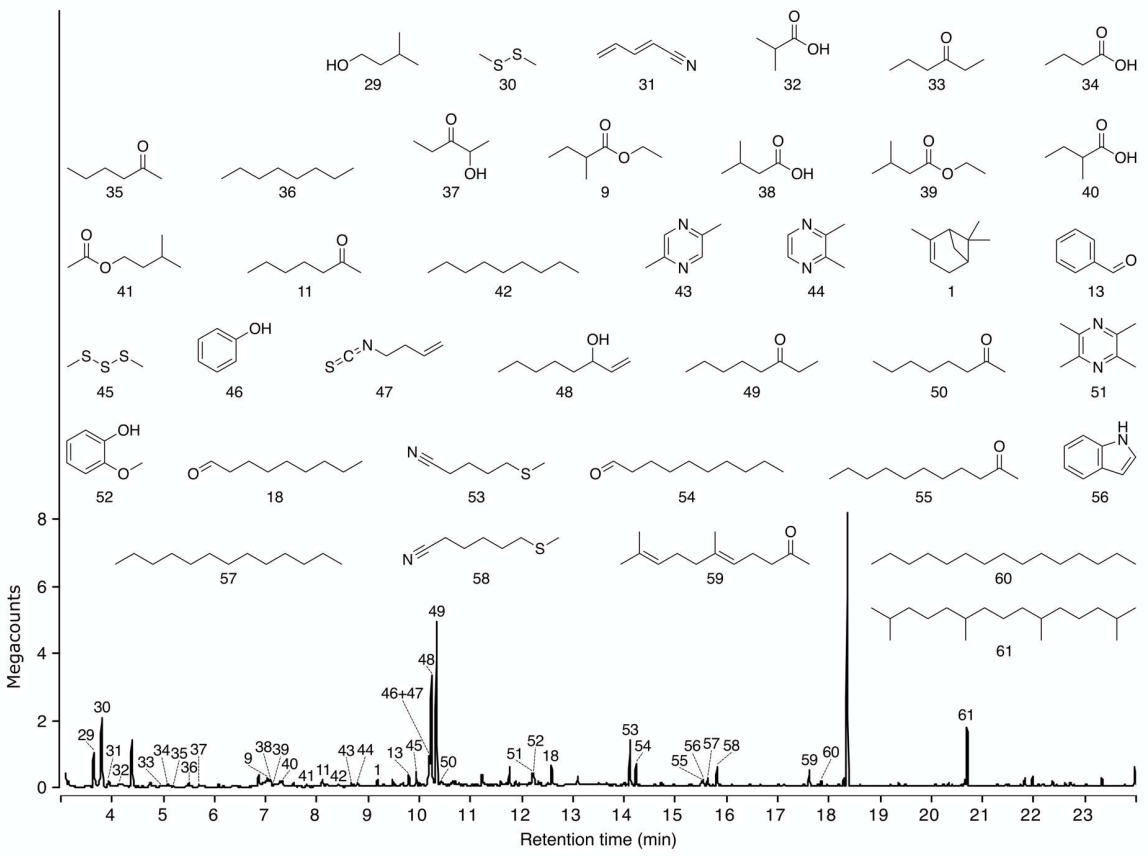

Figure 4.4. Total ion chromatogram of headspace volatile extracts (HVEs) of excreta from Ruffs, *Calidris pugnax*. Headspace volatiles were adsorbed on Porapak Q (Figure 4.1) and desorbed with pentane/ether (1/1). HVEs were analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 7890B GC coupled to a 5977A Series MSD). Names of compounds are listed in Table 4.2.

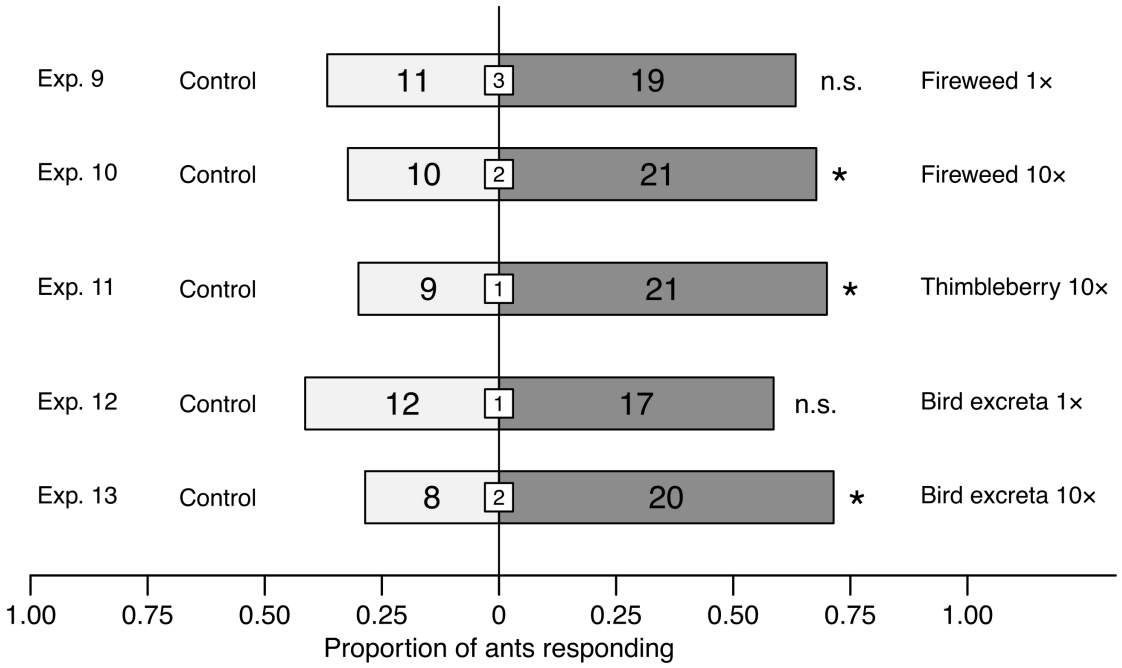

Figure 4.5. **Effect of headspace volatile extracts (HVEs) of fireweed, thimbleberry, and bird excreta on responses of *Camponotus modoc* worker ants in Y-tube olfactometer (Figure 4.1) experiments 9–12, each with 28–31 responding ants. HVEs in pentane/ether (1/1) were tested at doses equivalent (1×) to volatiles released from natural sources (Exps. 3, 4, 8) or 10-fold higher (10×). The same volume of pentane/ether (1/1) served as control stimulus. Numbers within bars indicate the number of ants responding to test stimuli and numbers within white inset boxes indicate the number of non-responding ants. Asterisks denote a significant preference for a test stimulus (Pearson's χ^2 tests: $p < 0.05$; n. s., not significant).**

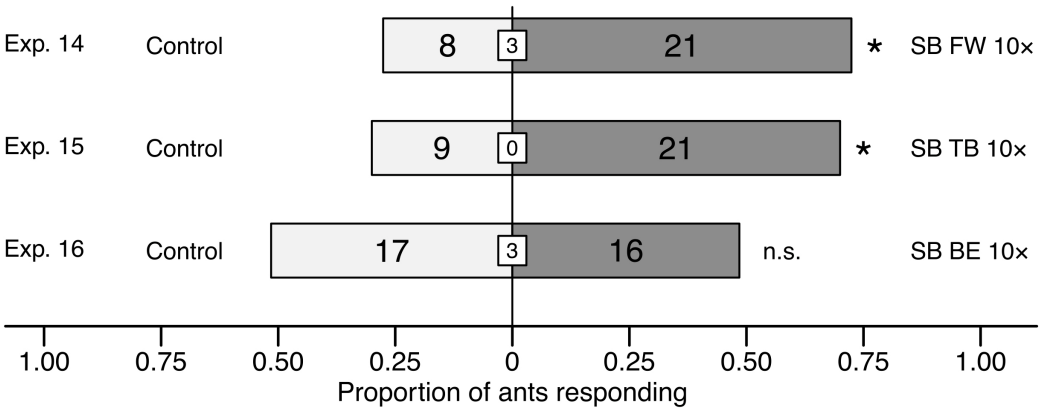

Figure 4.6. **Effect of synthetic blends (SB) of fireweed (FW), thimbleberry (TB), and bird excreta (BE) on responses of *C. modoc* worker ants in Y-tube olfactometer (Figure 4.1) experiments 14–16, each with 29–33 responding ants. SBs were formulated in pentane/ether (1/1) and tested at doses equivalent (1×) to volatiles released from natural sources (Exps. 2, 3, 8) or 10-fold higher (10×). The same volume of pentane/ether (1/1) served as control stimulus. Numbers within bars indicate the number of ants responding to test stimuli and numbers within white inset boxes indicate the number of non-responding ants. Asterisks denote a significant preference for a test stimulus (Pearson's χ^2 tests: $p < 0.05$; n. s., not significant).**

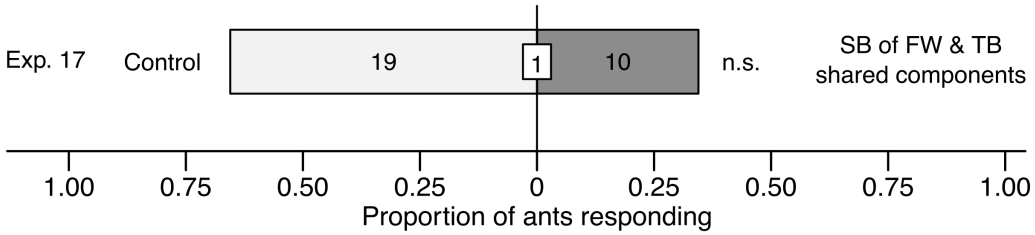

Figure 4.7. Effect of a synthetic blend (SB) comprising the two floral odorants [(*E*)-β-caryophyllene, α-humulene] shared between fireweed (FW) and thimbleberry (TB) on responses of *C. modoc* worker ants in Y-tube olfactometer (Figure 4.1) experiment 17. The SB was formulated in pentane/ether (1/1) and tested at a dose 10-fold higher (10×) than volatiles released from thimbleberry (Exp. 4). The same volume of pentane/ether (1/1) served as the control stimulus. Numbers within bars indicate the number of ants responding to test stimuli and the number within the white inset box indicates a single non-responding ant; n.s., not significant; p > 0.05.

Chapter 5.

Identification of the trail pheromone of the carpenter ant *Camponotus modoc*

A similar version of this chapter has been published: Renyard, A., Alamsetti, S. K., Gries, R., Munoz, A., and Gries, G. (2019). Identification of the trail pheromone of the carpenter ant *Camponotus modoc*. *J. Chem. Ecol.* 45, 901–913. doi:10.1007/s10886-019-01114-z.

5.1. Abstract

Trail pheromones deposited by ants lead nestmates to food sources. Based on previous evidence that the trail pheromone of the carpenter ant *Camponotus modoc* originates from the hindgut, our objective in this study was to identify the key component(s) of the pheromone. We collected *C. modoc* colonies from conifer forests and maintained them in an outdoor enclosure near our laboratory for chemical analyses and behavioral experiments. In gas chromatographic-electroantennographic detection and gas chromatography-mass spectrometric analyses of worker ant hindgut extracts, we identified five candidate components: 2,4-dimethylhexanoic acid, 2,4-dimethyl-5-hexanolide, pentadecane, dodecanoic acid and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin. In a series of trail-following experiments, ants followed trails of synthetic 2,4-dimethyl-5-hexanolide, a blend of the five compounds, and hindgut extract over similar distances, indicating that the hexanolide accounted for the entire behavioral activity of the hindgut extract. The hexanolide not only mediated orientation of *C. modoc* foragers on trails, it also attracted them over distance, indicating a dual function. Further analyses and bioassays with racemic and stereoselectively synthesized hexanolides revealed that the ants produce, and respond to, the (2*S*,4*R*,5*S*)-stereoisomer. The same stereoisomer is a trail pheromone component in several *Camponotus* congeners, indicating significant overlap in their respective trail pheromone communication systems.

5.2. Introduction

Ants are among the most successful groups of animals (Wilson 1987; Peeters and Ito 2015). They occupy nearly every terrestrial habitat (except polar regions), are

highly abundant, and have diverse and significant functions in their respective community and ecosystem (Hölldobler and Wilson 1990; Wilson and Hölldobler 2005). Ant colonies use sophisticated communication systems to effectively deploy their workforce for specific tasks such as cooperative brood care, nest defense and foraging, which are all dependent upon diverse chemical, tactile and vibratory signals (Hölldobler and Wilson 1990). Intraspecific chemical signals (pheromones) are paramount in ant communication (reviewed in Morgan 2008).

Many ants recruit nestmates to food sources, often using trails which require an array of pheromonal, tactile and motor signals to initiate and maintain (reviewed in Vander Meer 1998 and in Czaczkes et al. 2015). Foragers returning to their nest may use pheromonal signals, motor displays (e.g., lateral swaying of head and thorax, antennal drumming, rapid running) and trophallaxis for drawing their nestmates' attention and prompting them to follow trails to food sources (Hölldobler 1971; Hölldobler et al. 1974; Traniello 1977; Hölldobler and Wilson 1978; Vander Meer 1998; Hölldobler 1999; Czaczkes et al. 2015). Relatively persistent trail pheromone components serve as a chemical orientation guideline for nestmates (Vander Meer 1998; reviewed in Morgan 2009; Czaczkes et al. 2015).

When an ant scout that has fed on a newly discovered food source returns to the nest, she deposits a trail of message-bearing chemicals, termed trail pheromone (Morgan 2009). This trail pheromone provides a chemical guideline for her nestmates enabling them to efficiently locate the food source (Morgan 2009). As nestmates exploiting the food source also deposit trail pheromone, they reinforce the trail (Morgan 2009). Ants mark trails more strongly in response to abundant, high-quality food sources, and outgoing foragers more readily follow strong trails (Czaczkes et al. 2015). Therefore, trail pheromones not only guide ants to a food source, they are part of a dynamic communication system that helps the colony select the most profitable food sources and avoid exhausted ones (Czaczkes et al. 2015).

In general, ants from small nests forage on their own, whereas ants from large nests deploy pheromone trails to guide nestmates to food sources (Beckers et al. 1989; Planqué et al. 2010). Ants excrete trail pheromone from various exocrine glands (Morgan 2009). The characteristics of trail pheromone components seem to reflect the spatio-temporal distribution of the ants' food sources (Czaczkes et al. 2015). For

example, the black garden ant, *Lasius niger*, feeding heavily on honeydew from spatio-temporally stable aphid colonies (El-Ziady and Kennedy 1956), deploys a trail pheromone (3,4-dihydro-8-hydroxy-3,5,7-trimethylisocumarin) that is persistent (Bestmann et al. 1992; Kohl et al. 2003; Evison et al. 2008) but at low amount is not able to prompt trail-following with good accuracy (Evison et al. 2008; Grüter et al. 2011 but see Czaczkes et al. 2017). Conversely, the ant *Pheidole oxyops*, scavenging primarily on ephemeral dead insects (Czaczkes et al. 2011), deploys a highly volatile trail pheromone which nestmates follow with great accuracy (Czaczkes and Ratnieks 2012).

Carpenter ants in the genus *Camponotus* (Formicidae) are taxonomically diverse (>1,000 species) (Bolton 1995) and widely distributed over the globe (Janicki et al. 2016; Guénard et al. 2017). Carpenter ants recruit nestmates to food sources using pheromone trails, recruitment pheromones and motor displays (Hölldobler 1971; Hölldobler et al. 1974; Traniello 1977; Kohl 2001, 2003). For example, returning foragers of *C. pennsylvanicus* deposit pheromone from their poison gland that recruit nestmates to trails and trail pheromone from their hindgut that guides them on trails. Inside the nest, they use a waggle motor display coupled with rapid running intermissions to prompt nestmates to leave the nest and to follow trails (Traniello 1977). In carpenter ants, trail pheromone components are relatively persistent and typically consist of dihydroisocumarines and ∂-lactones (Morgan 2009; reviewed in Cerdá et al. 2014).

In the Pacific Northwest, the carpenter ant *Camponotus modoc* occurs in both forest and urban habitats (Hansen and Akre 1985; Raley and Aubry 2006). Nests may have as many as 50,000 nestmates (Akre et al. 1994) and can occupy multiple sites (polydomy) (Hansen and Akre 1985). Nests are active from April to September, with foraging activities taking place mostly at night (Hansen and Akre 1985). In forests, nests are often located in live trees but can also be present in stumps and logs (Hansen and Akre 1985). Ecological roles of carpenter ants in their respective communities are diverse. *Camponotus modoc* partners with aphids in mutualistic symbioses (Tilles and Wood 1982), scavenges arthropod prey (Hansen and Akre 1985; Tilles and Wood 1986), and – in turn – serves as prey for woodpeckers (Raley and Aubry 2006). In urban habitats, *C. modoc* readily infests human-made wooden structures, accounting for 78% of structural infestations in Washington (Hansen and Akre 1985). Despite the ecological and economic importance of *C. modoc,* there is relatively little known about its communication ecology.

Worker ants of *C. modoc* commute to food sources on trails which can extend up to 200 m away from their nest (David and Wood 1980; Hansen and Akre 1985). Workers navigate exploiting both visual and chemical foraging cues (David and Wood 1980). In bioassays, ants also followed experimentally deployed trails of hindgut extracts (Hansen and Akre 1985). Our research objectives were (1) to identify the trail pheromone components that mediate orientation behaviour of *C. modoc* foragers on trails, and (2) to determine whether the same pheromone component(s) attract foragers to trails.

5.3. Materials and methods

5.3.1. Ant rearing

We collected three *C. modoc* nests during the summer of 2016 and one nest during the summer of 2017 in conifer forests near Squamish, British Columbia. We harvested nests by cutting out infested log sections with a chainsaw (Husqvarna 394, 61 cm bar), placing them in large plastic bins (64 cm × 79 cm × 117 cm), and transporting them to the Science Research Annex (49º 16'33" N, 122º 54'55" W) of Simon Fraser University (SFU). In an outdoor undercover area of this Annex, the colonies were exposed to natural light and temperature cycles throughout the year. Each bin housing a nest was connected via clear Nalgene™ 180 (PVC) tubing (2.54 cm I.D.; Sigma-Aldrich, St. Louis, MO, USA) to a glass aquarium (51 cm × 28 cm × 30 cm) which served as the ants' foraging area. We provisioned the ants with blow flies, live mealworms, honey, apples, canned chicken, and 20% sugar water *ad libitum*.

5.3.2. Preparation of hindgut extracts

We collected worker ants into a glass holding tube (1.8 cm I.D. × 25 cm) and cold-euthanized them in a -15 °C freezer. We removed one ant at a time from the freezer and in distilled water excised her hindgut by cutting the alimentary canal where the Malpighian tubules enter and at the end of the rectum using fine forceps and dissection scissors (Fine Science Tools Inc., North Vancouver, BC, Canada) under a microscope [ZEISS, Oberkochen (formerly Jena), Germany]. We transferred the hindgut of 41 workers into a 4-mL glass vial (VWR, Radnor, PA, USA) containing dichloromethane (DCM; 500 μl; EMD Millipore Corp., Billerica, MA, USA) that was kept on ice during dissections. After macerating the hindguts with a glass rod, we kept the vial at room

temperature for 10 min to facilitate extraction. We then filtered the extract through glass wool in a glass pipette, using air pressure from a pipette bulb to transfer the extract into a clean 4-mL vial.

5.3.3. Analysis of hindgut extract by gas chromatographic-electroantennographic detection (GC-EAD)

We analyzed 2-µl aliquots (0.25 ant equivalents) of hindgut extract (final extract volume: ~330 µl (instead of 500 µl) due to some evaporation and pipette-to-vial-transfer losses) by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS), with procedures and equipment previously described in detail (Arn et al. 1975; Gries et al. 2002). Briefly, the GC-EAD set-up 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 · s^{-1}) with the following temperature program: 50° C for 1 min., 20° C · min^{-1} to 280° C. The injector port and flame ionization detector (FID) were set at 260 °C. For GC-EAD recordings ($N = 6$), we carefully pulled an antenna from a worker ant and suspended it between two glass capillary electrodes (1.0 × 0.58 × 100 mm; A-M Systems, Carlsborg, WA, USA) which we had adapted to accommodate an ant antenna and filled with a saline solution (Staddon and Everton 1980). We conservatively considered candidate pheromone components those odorants in hindgut extracts that elicited responses from five out of six antennae, taking into account that some antennae have a high sensitivity threshold to odorants.

5.3.4. Analysis of hindgut extract by GC-mass spectrometry (MS)

We analyzed candidate pheromone components on a Varian Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 MS column (30 m × 0.25 mm I.D.), using helium as the carrier gas (35 cm · s^{-1}). The injector port and ion trap were set at 250 °C and 200 °C, respectively, and the temperature program was as follows: 50 °C for 5 min, 10 °C · min^{-1} to 280 °C (held for 10 min). To identify candidate trail pheromone components in hindgut extract, we compared their retention indices (Van den Dool and Kratz 1963) relative to aliphatic alkanes and their mass spectra with those reported in the literature (Bestmann et al. 1995; Übler et al. 1995) and with those of authentic standards. To quantify the amount of candidate

pheromone components (see results) present in hindgut extracts of worker ants, we compared their GC peak integrations with those of synthetic standards, each prepared at various concentrations (1, 10, and 100 ng/µl). Based on results of these comparisons, we then proceeded to prepare and analyze additional authentic standards of known concentration until one GC peak integration matched that of the pertinent candidate pheromone component in hindgut extracts.

5.3.5. Absolute configuration of 2,4-dimethyl-5-hexanolide produced by *C. modoc*

2,4-Dimethyl-5-hexanolide is the key trail pheromone component of *C. modoc* (see Results) but *C. modoc* likely produces only one of the eight possible stereoisomers. We hypothesized that this is the (2*S*,4*R*,5*S*)-stereoisomer based on literature reports that other *Camponotus* species use it as a trail pheromone (Bestmann et al. 1999). To determine the absolute configuration of the 2,4-dimethyl-5-hexanolide produced by *C. modoc*, we ran hindgut extract, synthetic (racemic) 2,4-dimethyl-5-hexanolide and the synthetic (2*S*,4*R*,5*S*)-stereoisomer on a DB-5 column, comparing the mass spectra as well as the retention times of all compounds.

5.3.6. Purchase and syntheses of candidate trail pheromone components

2,4-Dimethylhexanoic acid, racemic 2,4-dimethyl-5-hexanolide, (2*S,*4*R,*5*S*)-dimethyl-5-hexanolide, and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocumarin were all synthesized in our laboratory (see Supplementary Information). Pentadecane and dodecanoic acid (lauric acid) with 99% and 98% purity, respectively, were purchased from Sigma-Aldrich.

General design of a circular trail bioassay To standardize visual cues during behavioural bioassays, we ran all bioassays under a metal scaffold (123 cm × 57 cm × 36 cm) enclosed in black fabric and illuminated from above with two 32 W fluorescent lights (121.92 cm; Philips, Amsterdam, Netherlands).

In preparation for bioassays, we detached the Nalgene tubing (see above) that connects a nesting bin to a foraging arena from the arena and plugged the tubing with a cotton ball. For each replicate, we isolated a single ant from a randomly selected bin. To accomplish this, we removed the cotton ball and inserted the tubing into a 15-mL Falcon tube (Fisher Scientific, Corning, NY, USA) with a hole (0.7 cm I.D.) cut in its tapered tip but plugged with cotton, thus enabling an ant to exit the Nalgene tubing and to enter the Falcon tube.

To prepare test stimuli, we used a micro-syringe to apply a continuous trail of hindgut extract or synthetic pheromone [1 ant equivalent dissolved in DCM (25 μL)], or a DCM control (25 μL), on the circumference of a circular Whatman filter paper (diam: 185 mm; Sigma-Aldrich, St. Louis, MO, USA) marked with pencil in 1-cm intervals. To initiate a bioassay, we placed the filter paper in the center of a plexiglass arena (64 cm × 44 cm × 10 cm or 51 cm × 36 cm × 8 cm) and positioned a Falcon tube containing a single ant so that its tapered tip laid flush with the arena floor and its exit hole was 2.5 cm away from the edge of the filter paper. We then removed the cotton plug from the Falcon tube, thus enabling the ant to calmly walk into the bioassay arena. Once an ant had exited the tube, we video-recorded her trail-following response for 5 min, using a Canon FS300 video camera (Canon, Tokyo, Japan), or a Sony HDR-CX210 (Sony, Tokyo, Japan), mounted overhead the bioassay arena.

We reviewed the video footage in slow motion using VLC Media Player (Version 2.2.6) or QuickTime Player (Version 10.4) to count the number of 1-cm intervals a trail-following ant had crossed during the bioassay, allowing us to determine the total distance she had covered as a measure of her orientation behavior in response to the test stimulus [Morgan 2009; see Supplementary Video (excerpt)]. For all experiments, we ran treatment and control stimuli in parallel. In each experiment, we considered the few (0-5) ants not exiting a Falcon tube within 10 min non-responders and excluded them from statistical analyses. After each replicate, we wiped the arena and countertops with 70% ethanol and hexane.

5.3.7. Specific experiments

Exp. 1: Effect of hindgut extract on trail-following responses of ants

To assess the effect of hindgut extract (hypothesized to contain trail pheromone) on the trail-following responses of ants, for each replicate we applied a trail of either hindgut extract (1 ant equivalent in 25 μL of DCM; n = 20) or a DCM control (25 μL; n = 20) to the circumference of a circular filter paper (see general design), video recording the distance each ant followed.

Exp. 2: Comparative effects of hindgut extract and synthetic blends of trail or alarm pheromone components on trail-following responses of ants

As hindgut extract elicited persistent trail-following behavior (see Results), we wanted to determine whether a synthetic blend (SB) of candidate trail pheromone components identified in hindgut extracts ("SB-Trail pheromone") (see below) elicited comparable trail-following responses. Moreover, to ascertain that the trail-following response was prompted by trail pheromone present in the hindgut, and not just by any other pheromone present in exocrine glands, we also prepared a synthetic blend of candidate alarm recruitment pheromone components present in the poison gland ("SB-Poison gland"; Renyard et al., manuscript under peer review). We tested four treatments (n = 20 each) (Table 5.1): (1) hindgut extract (1 ant equivalent); (2) "SB-Trail pheromone" (1 ant equivalent) containing racemic 2,4-dimethylhexanoic acid, racemic 2,4-dimethyl-5-hexanolide, dodecanoic acid, and pentadecane; (3) "SB-poison gland" (1 ant equivalent) containing undecane, tridecane, pentadecane, heptadecane, (Z)-7-pentadecene, (Z)-8-heptadecene, (Z)-7-heptadecene, hexadecan-1-ol, hexadecyl formate, hexadecyl acetate, benzoic acid, formic acid (Table 5.1); and (4) a solvent (DCM) control. SB-Trail pheromone lacked 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocumarin which elicited antennal responses in GC-EAD analyses of hindgut extract (see Results) but could not be synthesized in time for this bioassay. For bioassays, we formulated synthetic blends according to the amount and ratio of EAD-active candidate pheromone components present in hindgut and poison gland extracts (see Table 5.1).

Exp. 3: Determining the essential component(s) in the trail pheromone blend

To determine the essential component(s) in the trail pheromone blend, we tested at 1 ant equivalent (1) the complete blend [racemic 2,4-dimethylhexanoic acid, racemic 2,4-dimethyl-5-hexanolide, dodecanoic acid, pentadecane, and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocumarin (which was previously missing in experiment 2)] (n = 20), and (2-5) partial blends (PBs) (n = 20 each) that lacked one or two components such as 2,4-dimethyl-5-hexanolide (2), both 2,4-dimethylhexanoic acid and dodecanoic acid (3), pentadecane (4), or 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocumarin (5).

Exp. 4: Effect of trail pheromone dose on trail-following responses of ants

To determine the amount of pheromone needed to trigger trail-following responses, we ran a dose-response experiment (Table 5.1), testing the complete blend of candidate trail pheromone components at ant equivalents of 0.01 (n = 16), 0.1 (n = 19), 1.0 (n = 15) and 10 (n = 18), for the trail-following responses of ants. For each dose tested, DCM served as the control stimulus (n = 19).

Exp. 5: Comparative effects of the complete candidate trail pheromone blend, (racemic) 2,4-dimethyl-5-hexanolide and the (2S,4R,5S)-stereoisomer on the trail-following responses of ants

To ascertain that (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide is the key trail pheromone component of *C. modoc*, we compared the effects of the complete candidate trail pheromone blend (containing racemic 2,4-dimethyl-5-hexanolide), racemic 2,4-dimethyl-5-hexanolide and the (2*S*,4*R*,5*S*)-stereoisomer on the trail-following responses of ants (Table 5.1). We tested each of the three synthetic stimuli (n = 20 each) at one ant equivalent, making sure that the amount of the (2*S*,4*R*,5*S*)-stereoisomer (4 ng) was identical in all test stimuli (n = 20).

Exp. 6: Trail-following of ants in response to (2S,4R,5S)-2,4-dimethyl-5-hexanolide in the absence of a physical edge

Carpenter ants tend to follow edges and grooves (Hansen and Akre 1985; Klotz and Reid 1992). To ascertain that *C. modoc* orients on pheromone trails not only in the presence of a physical edge, such as the rim of filter paper in preceding experiments, but also in the absence of any edge, we tested the effect of (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide in an “edgeless” experimental design. On a sheet (21.59 × 27.94 cm) of

white printer paper (EarthChoice30; Domtar Corp., Fort Mill, SC, USA), we drew two pencil lines (each 25 cm long) marked at 1-cm intervals and divergent in a V-shape at a 45° angle. Using a micro-syringe, we applied the treatment stimulus [(2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide in 20 μl of DCM] at the same concentration (69 pg/cm) as tested in circular trail bioassays or the control stimulus (20 μl of DCM) by random assignment to one of the two lines. We then placed this paper in the bioassay arena such that the tip of the Falcon tube containing a bioassay ant (see above) resided at the convergent point of the "V". We allowed each ant 10 min to exit the Falcon tube, and for 2.5 min then video-recorded the number of 1-cm intervals she crossed on each line.

Exp. 7: Anemotactic attraction of ants in Y-tube olfactometers to (2S,4R,5S)-2,4-dimethyl-5-hexanolide

To test whether (2*S,*4*R*,5*S*)-2,4-dimethyl-5-hexanolide not only mediates orientation behaviour of *C. modoc* foragers on trails (see Results) but also attracts them to trails, we tested the effect of (2*S,*4*R*,5*S*)-2,4-dimethyl-5-hexanolide in Pyrex glass Y-tube olfactometers (inner diameter: 2.5 cm, main stem: 22.5 cm, side arms: 19 cm, angle of arms: 120°; see Fig. 1C in Derstine et al. 2017). To bait olfactometers, we placed a 1-cm long piece of braided cotton roll (Richmond Dental and Medical, NC, U.S.A) at the orifice of each side arm and by random assignment treated the roll with either 4 ng (1 ant equivalent) of (2*S,*4*R*,5*S*)-2,4-dimethyl-5-hexanolide in 12 μl of DCM or a 12-μl DCM control. For each replicate, we allowed a single ant from a randomly selected colony to calmly walk into a glass holding tube (25 cm × 1.8 cm inner diameter). To commence a bioassay, we connected the holding tube to the Y-tube stem via a ground male/female glass joint and attached the holding tube to a Neptune Dyna pump (A.O. Smith, Tipp City, OH, USA), drawing air at a rate of 0.5 L/min towards an ant entering the Y-tube. We recorded an ant's first choice when she crossed a mark 6 cm from the orifice of a Y-tube arm, and we considered all ants making no choice within 10 min non-responders, which we excluded from statistical analyses. We used a clean Y-tube and holding tube for each replicate, cleaning them with hot water and soap (Sparkleen, Thermo Fisher Scientific, MA, U.S.A) and drying them in an oven at 100 °C for at least 1 h.

5.3.8. Data analyses

We analysed data and produced graphics using R (version: 3.2.2) and RStudio (version: 1.0.136) (R Core Team 2018). For experiments 1-5, we compared the effect of various pheromone treatments on the mean number of one-centimetre intervals followed by ants using generalized linear models (GLM) with a quasi-Poisson error structure. In experiments 2, 4 and 5, we used a Tukey's HSD test for multiple comparisons of mean distance followed between treatment groups. For experiment 3, we analysed data using the GLM making *a priori* contrasts between the complete trail pheromone blend and the various partial blends. For experiment 6, we compared the effects of (2*S,*4*R*,5*S*)-2,4-dimethyl-5-hexanolide and a solvent control on the distance followed by ants using a paired t-test. For experiment 7, we analyzed first-choice responses of worker ants in Y-tube olfactometers using a χ^2 test.

5.4. Results

5.4.1. Analyses of hindgut extract by GC-EAD and GC-MS

Six compounds in hindgut extract consistently elicited antennal responses from worker ants in GC-EAD analyses (Fig. 5.1). We identified these candidate trail pheromone components as 2,4-dimethylhexanoic acid (4 ng; mean amount per ant), 2,4-dimethyl-5-hexanolide (4 ng), pentadecane (27 ng), dodecanoic acid (15 ng), diethyl phthalate, and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (4 ng).

5.4.2. Absolute configuration of 2,4-dimethyl-5-hexanolide produced by *C. modoc*

The eight isomers present in racemic 2,4-dimethyl-5-hexanolide eluted on a DB-5 column as three separate peaks, with the largest peak containing six isomers (Fig. 5.2, middle). Synthetic (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide (Fig. 5.2, bottom), the first-eluting stereoisomer in racemic 2,4-dimethyl-5-hexanolide, and a natural compound in hindgut extracts of *C. modoc* (Fig. 5.2, top)*,* all had both identical mass spectra, and retention times on a DB-5 column (Fig. 5.2). These data support the conclusion that *C. modoc* produces the (2*S*,4*R*,5*S*)-stereoisomer of 2,4-dimethyl-5-hexanolide.

5.4.3. Exp. 1: Effect of hindgut extract on trail-following responses of ants

The mean distance followed by worker ants on hindgut extract trails was 3.4 times longer than the distance they followed solvent control trails (quasi-Poisson GLM, d.f. = 38, t = 3.854 $P < 0.001$; Fig. 5.3).

5.4.4. Exp. 2: Comparative effects of hindgut extract and synthetic blends of candidate trail or alarm pheromone components on trail-following responses of ants

The distances worker ants followed trails of (*i*) hindgut extract, (*ii* + *iii*) synthetic blends of trail or alarm pheromone components, or (*iv*) a solvent control differed significantly (ANOVA, $F_{[3, 76]} = 13.878$, $P < 0.0001$; Fig. 5.4). Ants followed hindgut extract trails, or synthetic trail pheromone trails (lacking isocumarin) over a longer distance than they followed synthetic alarm pheromone trails (hindgut extract: Z = 3.194, $P = 0.007$; SB-Trail pheromone: Z = 3.457, $P = 0.003$) and solvent control trails (hindgut extract: Z = 4.544, $P < 0.0001$; SB-Trail pheromone: Z = 4.745, $P < 0.0001$). Ants walked similar distances in response to hindgut extract trails and synthetic trail pheromone trails (Z = 0.301, $P = 0.99$), and in response to synthetic alarm pheromone trails and solvent control trails (Z = 1.896, $P = 0.22$).

5.4.5. Exp. 3: Determining the essential component(s) in the trail pheromone blend

The distances ants followed synthetic trail pheromone trails were contingent upon the composition of the trail pheromone blend. Ants followed trails for a shorter distance when 2,4-dimethyl-5-hexanolide was absent from the blend ($t = -2.103$, $P = 0.038$; Fig. 5.5). Equivalent effects did not arise when other components were omitted from the blend, such as acids ($t = -0.256$ $P = 0.80$), pentadecane ($t = 0.574$, $P = 0.57$) or isocumarin ($t = 0.090$, $P = 0.93$).

5.4.6. Exp. 4: Effect of trail pheromone dose on trail-following responses of ants

The distances worker ants followed a synthetic trail pheromone trail was affected by the amount of pheromone [0.01, 0.1, 1.0 or 10 ant equivalents (AE)] applied ($F_{[4, 82]}$ = 11.716, P < 0.0001; Fig. 5.6). Ants followed pheromone trails of 0.01, 0.1, and 1.0 AEs for a longer distance than they followed control trails (0.01 AE: Z = 3.045 P = 0.019; 0.1 AE: Z = 5.282, P < 0.0001; 1.0 AE: Z = 5.326, P < 0.0001). The distance ants followed pheromone trails of 10 AEs or solvent control trails did not differ (Z = 2.701 P = 0.052). Ants followed trails of 0.1 and 1.0 AEs for a longer distance than they followed trails of 10 AEs (0.1 AE: Z = -2.998, P = 0.022; 1.0 AE: Z = -3.093 P = 0.016) but followed trails of 0.01 and 10 AEs for the same distance (Z = -0.432 P = 0.99). Trails of 0.01 AEs prompted ants to follow the same distance as trails of either 0.1 or 1.0 AEs (0.1 AE: Z = 2.484, P = 0.091; 1.0 AE: Z = 2.598, P = 0.077), as did trails of either 0.1 or 1.0 AEs (Z = 0.239, P = 0.999).

5.4.7. Exp. 5: Comparative effects of the complete candidate trail pheromone blend, (racemic) 2,4-dimethyl-5-hexanolide and the (2*S*,4*R*,5*S*)-stereoisomer on the trail-following responses of ants

The distances worker ants followed trails of (*i*) the complete trail pheromone blend, (*ii*) racemic 2,4-dimethyl-5-hexanolide, (*iii*) the (2*S*,4*R*,5*S*)-stereoisomer, and (*iv*) a solvent control differed significantly ($F_{[3,76]}$ = 6.8283, P < 0.001; Fig. 5.7). Ants followed trails of the former three treatment trails for a longer distance than they followed a solvent control trail (racemic 2,4-dimethyl-5-hexanolide: Z = 3.496, P = 0.003; (2*S*,4*R*,5*S*)-stereoisomer: Z = 3.753, P = 0.001; complete blend: Z = 3.507, P = 0.002). Ants walked the same distance on a trail of racemic 2,4-dimethyl-5-hexanolide, the (2*S*,4*R*,5*S*)-stereoisomer, or the complete trail pheromone blend (pairwise comparisons: racemic 2,4-dimethyl-5-hexanolide *vs* (2*S*,4*R*,5*S*)-stereoisomer: Z = 0.314, P = 0.99; racemic 2,4-dimethyl-5-hexanolide *vs* complete blend: Z = -0.013, P = 1.00; (2*S*,4*R*,5*S*)-stereoisomer *vs* complete blend: Z = 0.301, P = 0.99).

5.4.8. Exp. 6: Trail-following responses of ants to (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide in the absence of a physical edge

When presented with a choice between two pencil lines drawn in divergent V-shape in the center of a printer paper and treated with either (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide or a solvent control, worker ants followed the hexanolide-treated line for a 21-fold longer distance than the control line (Paired t-test, d.f. = 19, t = -5.0619, $P < 0.0001$; Fig. 5.8).

5.4.9. Exp. 7: Anemotactic attraction of ants in Y-tube olfactometers to (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide

In two-choice Y-tube olfactometers, 16 ants chose the side arm baited with the hexanolide, whereas four ants chose the side arm treated with a solvent control ($\chi^2 = 7.2$, d.f. = 1, p = 0.007; Fig. 5.9).

5.5. Discussion

(2*S*,4*R*,5*S*)-2,4-Dimethyl-5-hexanolide is an essential, and possibly the only, trail pheromone component of *C. modoc* that attracts foragers to trails and that mediates their orientation on trails. Below, we elaborate on the chemical analyses and the behavioral bioassays that led to this conclusion.

Worker ants of *C. modoc* readily followed trails of hindgut extracts (Hansen and Akre 1985; this study, Fig. 5.3), indicating that trail pheromone originates, at least in part, from the hindgut (or rectal sac). As we dissected ant hindguts in water, it is conceivable that an additional (highly polar) pheromone component may have remained in the water. Yet, ants followed trails of hindgut extract for up to 1,649 cm, revealing a strong fidelity to trail pheromone (propensity to follow and stay on the trail). Trail-following behavior to hindgut extract has previously been demonstrated in several genera of formicine ants including *Paratrechina* (Blum and Wilson 1964; Witte et al. 2007), *Oecophylla* (Hölldobler and Wilson 1978), *Myrmelachista* (Blum and Wilson 1964), *Anoplolepis* (Lizon à l'Allemand and Witte 2010), *Polyergus* (Visicchio et al. 2001), and *Polyrhachis* (Liefke et al. 2001) but pheromone components have been identified only in *Camponotus*, *Formica* and *Lasius* (Morgan 2009; Cerdá et al. 2014).

GC-EAD analyses of *C. modoc* hindgut extract revealed six components that consistently elicited responses from worker ant antennae: 2,4-dimethylhexanoic acid, 2,4-dimethyl-5-hexanolide, pentadecane, dodecanoic acid, diethylphthalate, and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (Fig. 5.1). We considered the phthalate a contaminant but the five other compounds candidate pheromone components. The initial synthetic blend of candidate pheromone components lacked 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin but was still as effective as hindgut extract, indicating that all essential pheromone components were present in that blend (Fig. 5.4). Testing the complete 5-component blend and partial blends (lacking one or two components) for the trail-following responses of ants revealed that the 2,4-dimethyl-5-hexanolide was the key component (Fig. 5.5).

To determine the absolute configuration of this hexanolide, we analyzed hindgut extract of ants, racemic 2,4-dimethyl-5-hexanolide, and stereospecifically synthesized (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide by GC-MS. We selectively synthesized the (2*S*,4*R*,5*S*)-stereoisomer for these analyses because it was the only isomer to elicit trail-following in other *Camponotus* species (Bestmann et al. 1999). Our analyses showed that the (2*S*,4*R*,5*S*)-stereoisomer, the first eluting stereoisomer in racemic 2,4-dimethyl-5-hexanolide, and compound 7 in *C. modoc* hindgut extract, all had identical retention times (Fig. 5.2) and mass spectra, demonstrating that C. modoc produces (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide, which is the most thermodynamically stable of the eight possible stereoisomers (Bestmann et al. 1999).

To confirm that the (2*S*,4*R*,5*S*)-stereoisomer was indeed the essential trail pheromone component of C. modoc, we tested the trail-following behavior of ants in response to the complete trail pheromone blend (containing racemic 2,4-dimethyl-5-hexanolide), racemic 2,4-dimethyl-5-hexanolide, and the (2*S*,4*R*,5*S*)-stereoisomer. Our findings that ants walked the same distance on each of these trails (Fig. 5.7) demonstrate that (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide is likely the single-component trail pheromone of *C. modoc* that mediates orientation behavior on trails. We offer this tentative conclusion, acknowledging that we took an indirect approach (Choe et al. 2012) to identify the pheromone, extracting the hindgut instead of the trail deposited by ants which can consist of multiple components (Attygalle and Morgan 1983; Billen et al. 1992; Janssen et al. 1997; Attygalle et al. 1998) from different exocrine glands (Jackson et al. 1989; Hölldobler et al. 1994; Janssen et al. 1995). As a result, we may have missed

additional components originating from sources other than the hindgut. Analysis of in-situ trails of the Argentine ant, *Linepithema humile*, revealed two new pheromone components that originate from the pygidial gland (Choe et al. 2012). For *C. modoc*, however, exocrine gland components are not likely to improve orientation behavior. Synthetic poison gland constituents, e.g., that typically induce alarm responses did not elicit any trail-following behavior by *C. modoc* (Fig. 5.4) when tested at 1 ant equivalent (Table 5.1). Similarly, formic acid alone, as a major poison gland constituent, prompted imprecise and inconsistent trail-following behavior by *C. socius* and *C. sericeiventris*, but it did serve as a strong recruitment signal to trails (Kohl et al. 2001, 2003).

As shown in multiple ant species, the amount of trail pheromone greatly affects the trail-following response of nestmates (Evershed et al. 1982; Kohl et al. 2001, 2003; Morgan et al. 2006). Workers of *C. modoc* responded to synthetic trail pheromone amounting to only 0.01 ant equivalents, which approximates 0.7 pg of pheromone per 1 cm of trail. They followed trails of 0.1 and 1.0 AE for the longest distance and responded equally poorly to 10-AE trails and solvent control trails (Fig. 5.6). Comparable results were obtained in studies with *Myrmica rubra* and *Atta sexdens sexdens* where worker ants walked shorter distances on high-dose pheromone trails (Evershed et al. 1982; Morgan et al. 2006). Sensory overload or saturation is a potential explanation for this phenomenon, but the underlying mechanisms have yet to be thoroughly studied. It is noteworthy that ants deposit less trail pheromone in response to collisions with nestmates on trails (Czaczkes et al. 2013b) and to heavily marked trails (Czaczkes et al. 2013a), apparently modulating traffic levels on trails to optimize food transport to their nest (Fourcassié et al. 2010). Findings that workers of other *Camponotus spp*. readily followed trails of as many as ~100 AEs (Kohl et al. 2001, 2003) seem surprising but can be attributed to the binary-choice type bioassay used in these studies. When offered a choice, ants are more likely to select a pheromone trail than a solvent control trail and are more likely to select a trail that is more strongly marked (Czaczkes et al. 2015). Choice bioassays typically reveal the preference for one of two test stimuli (Morgan 2009), whereas circular trail bioassays, as used in our study, reveal the behavioral activity of the one stimulus being tested (Morgan 2009).

In circular trail bioassays (Figs. 5.3, 5.4, 5.6, 5.7), ants followed pheromone trails for a significantly longer distance than they followed solvent control trails. Ants apparently responding to solvent control trails may have somewhat been guided by the

edge of the filter paper disc. This interpretation is supported by observation that foraging workers of both *C. modoc* and *C. pennsylvanicus* follow physical guidelines such as edges and grooves (Hansen and Akre 1985; Klotz and Reid 1992), a behaviour which may help conserve energy and save foraging time (Klotz et al. 2000). Noteworthy, workers of *C. modoc* readily followed trails of synthetic 2,4-dimethyl-5-hexanolide irrespective of trail pheromone application through the centre of bioassay paper (Fig. 5.8) or along its edge (Figs. 5.4, 5.6, 5.7), indicating strong fidelity to synthetic trail pheromone even in the absence of a physical guideline. Moreover, (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide not only mediates orientation behaviour of *C. modoc* foragers on trails (Figs. 7, 8), it also attracts them to trails (Fig. 5.9), indicating a dual function of the trail pheromone, which was not previously known in formicine ants.

Sympatric ant species may encounter each other's trails, and some have overlapping pheromone components. For example, *C. herculeanus* and *L. niger* each share 2,4-dimethyl-5-hexanolide and 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin as trail pheromone components with *C. modoc*. Behavioral responses to hindgut extract of heterospecific ants has been demonstrated in laboratory experiments. *Camponotus silvicola* follow trails of *C. rufipes* hindgut extract (Übler et al. 1995), and *C. silvicola* and *L. niger* that share 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin as a trail pheromone component follow hindgut extracts of either species (Bestmann et al. 1992; Übler et al. 1995). Ants that eavesdrop on pheromone trails of heterospecifics gain information on the location of profitable food sources (Wilson 1965; Adams 1990; Gobin et al. 1998; Menzel and Blüthgen 2010; Menzel et al. 2010). For example, C. beebei and *C. rufifemur* follow trails of *Azteca chartifex* and *Crematogaster modiglianii*, respectively (Wilson 1965; Menzel et al. 2010). As ants often respond aggressively to non-nestmates, one might wonder about the underlying mechanisms that allow sympatric ants to avoid conflict on shared trails. Temporal partitioning of communication channels and foraging activities is one such underlying mechanism. For example, *C. pennsylvanicus* and *F. subsericea* forage on the same aphid-infested trees but have opposite diel foraging schedules (Klotz 1984). Similarly, workers of *C. beebei* follow trails of *A. charifex* during the day when *Azteca* ants are mainly resting (Wilson 1965).

The responsiveness of worker ants to a task-allocating stimulus, such as trail-following, is dependent upon their physiological status (hunger, age, caste), prior experience (e.g., social interactions) and stimulus strength (Kohl et al. 2001, 2003;

Morgan et al. 2006; Kleineidam et al. 2007; Muscedere et al. 2012; Czaczkes et al. 2015). In our experiments, the responsiveness of *C. modoc* workers to trail pheromone differed considerably due likely to intrinsic factors. Our experimental response variable, the distance a worker ant followed a trail in 5 min, was dependent upon her walking speed and trail fidelity. In other studies, outbound foragers of the leafcutter ant *Acromyrmex lundi* walked faster when they had trophallaxed with a nestmate that recently fed on a rich food source (Roces 1993); minor and major workers of *A. s. sexdens* followed trails with greater fidelity than medium workers (Morgan et al. 2006); and aging minor workers of *Pheidole dentata* sensorially became more responsive to task-allocating stimuli including trail pheromone (Seid and Traniello 2006; Muscedere et al. 2012). In *Camponotus*, worker task-allocation appears to be age- and caste-related (Mersch et al. 2013; Simola et al. 2016). In *C. modoc* specifically, smaller workers forage, whereas majors mostly remain in the nest vicinity (Tilles and Wood 1986). While we predominantly selected minor and medium workers for testing, we had no knowledge of their age which could explain their variable responsiveness to trail pheromone.

In conclusion, our study shows that (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide is an essential trail pheromone component of *C. modoc*. As shown for the invasive Argentine ant, short-lived trail recruitment pheromone components have the potential to improve the effectiveness of poisonous food baits as a control tactic for ants. Admixture of trail pheromone to lethal baits for Argentine ants increased bait consumption and ant mortality, and reduced ant activity in the field (Greenberg and Klotz 2000; Welzel and Choe 2016). Synthetic (2*S*,4*R*,5*S*)-2,4 dimethyl-5-hexanolide could be tested on *C. modoc* and congeners for comparable control effects.

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5.8. Tables

Table 5.1. Objectives (O) and stimuli tested for trail-following responses of *Camponotus modoc* ants

Exp. #	Test stimuli (T)	Number of replicates
O_1: Assess trail-following responses of ants to hindgut extract		
1	T_1: Hindgut extract; T_2: Solvent control	T_1 - T_2: 20,20
O_2: Identify candidate trail pheromone components in hindgut extract		
O_3: Compare trail-following responses to synthetic blends of trail pheromone and alarm pheromone		
2	T_1: Hindgut extract; T_2: SB–Trail pheromone[ab]; T_3: SB–Poison gland[ac]; T_4: Solvent control	T_1 - T_4: 20,20,20,20
O_4: Determine the essential trail pheromone components		
3	T_1: SB–Trail pheromone[ab]; T_2: SB *minus* hexanolide; T_3: SB *minus* acids; T_4: SB *minus* pentadecane; T_5: SB *minus* isocumarin	T_1 - T_5: 20,20,20,20,20
O_5: Determine the amount of trail pheromone needed to trigger trail-following		
4	T_1: SB – 0.01 ant equivalents (AE); T_2: SB – 0.1 AE; T_3: SB – 1.0 AE; T_4: SB – 10 AE; T_5: Solvent control	T_1 - T_5: 16,19,15,18,19
O_6: Identify the stereoisomer of 2,4-dimethyl-5-hexanolide present in hindguts extract		
O_7: Determine the key stereoisomer of 2,4-dimethyl-5-hexanolide		
5	T_1: SB–Trail pheromone; T_2: Racemic 2,4-dimethyl-5-hexanolide; T_3: (2*S*,4*R*,5*S*)-Stereoisomer; T_4: Solvent control	T_1 - T_4: 20,20,20,20
O_8: Determine trail-following responses to trail pheromone in the absence of a physical edge		
6	T_1: (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide; T_2: Solvent control	T_1 - T_2: 20,20
O_9: Determine anemotactic attraction of ants to trail pheromone		
7	T_1: (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide; T_2: Solvent control	T_1 - T_2: 20,20

[a] Hindgut extract; synthetic blend (SB)–Trail pheromone and SB–Poison gland were both tested at one ant equivalent

[b] SB–Trail pheromone: 2,4-dimethylhexanoic acid (4 ng), 2,4-dimethyl-5-hexanolide (32 ng), pentadecane (27 ng), dodecanoic acid (15 ng), 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin (4 ng); Note: SB–Trail pheromone in experiment 2 lacked the isocumarin which was still being synthesized.

[c] SB–Poison gland: undecane (20 ng), tridecane (10 ng), pentadecane (3 ng), heptadecane (1 ng), (Z)-7-pentadecene (1 ng), (Z)-8-heptadecene (1 ng), (Z)-7-heptadecene (1 ng), hexadecan-1-ol (20 ng), hexadecyl formate (30 ng), hexadecyl acetate (2 ng), benzoic acid (12 ng), formic acid (10 µg

5.9. Figures

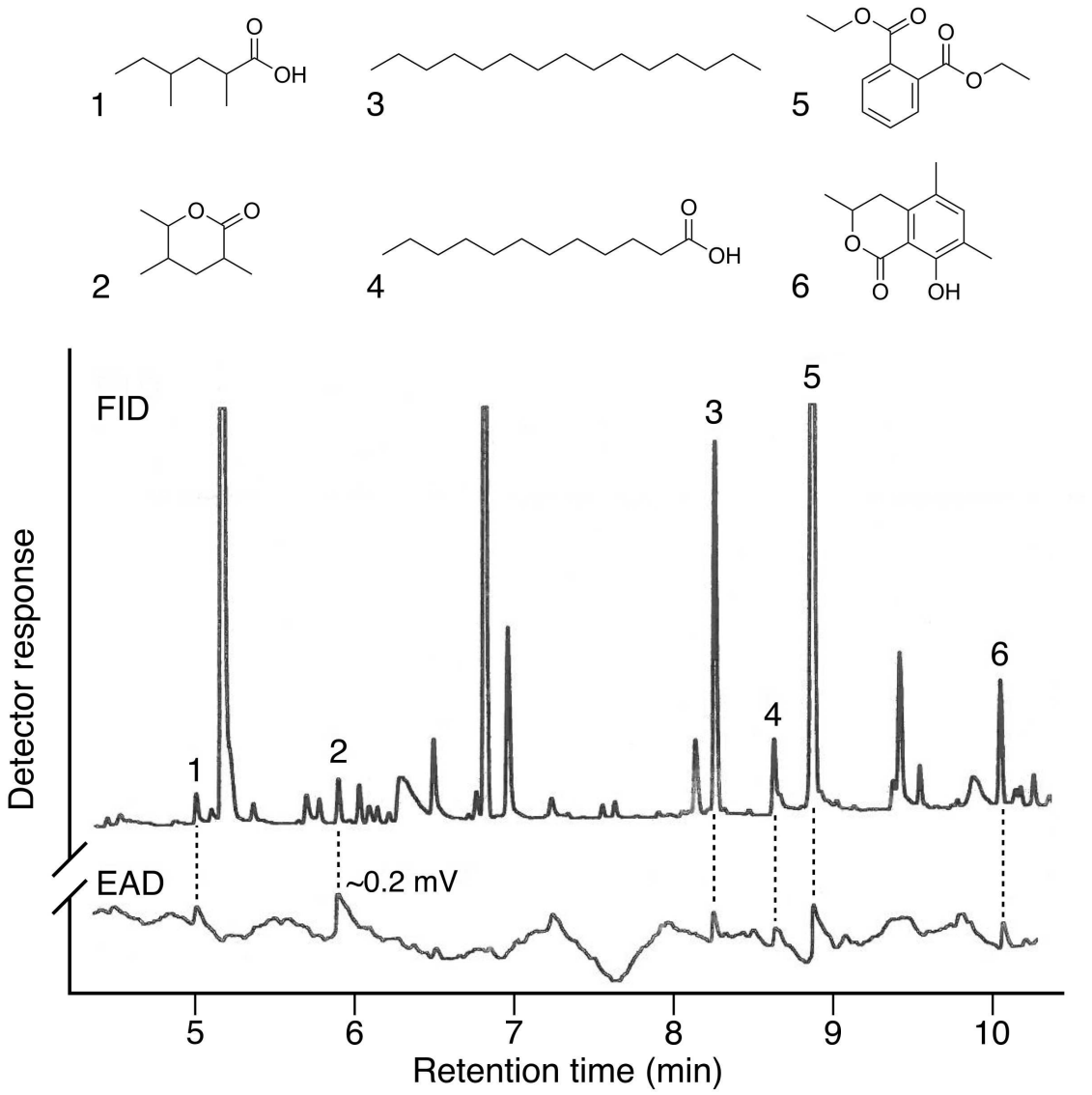

Figure 5.1. **Representative recording (n = 6) of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: antenna of a *Camponotus modoc* worker ant) to aliquots of *C. modoc* worker ant hindgut extract. The six components that consistently elicited antennal responses were identified as: (1) 2,4-dimethylhexanoic acid, (2) 2,4-dimethyl-5-hexanolide, (3) pentadecane, (4) dodecanoic acid, (5) diethylphthalate, and (6) 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin.**

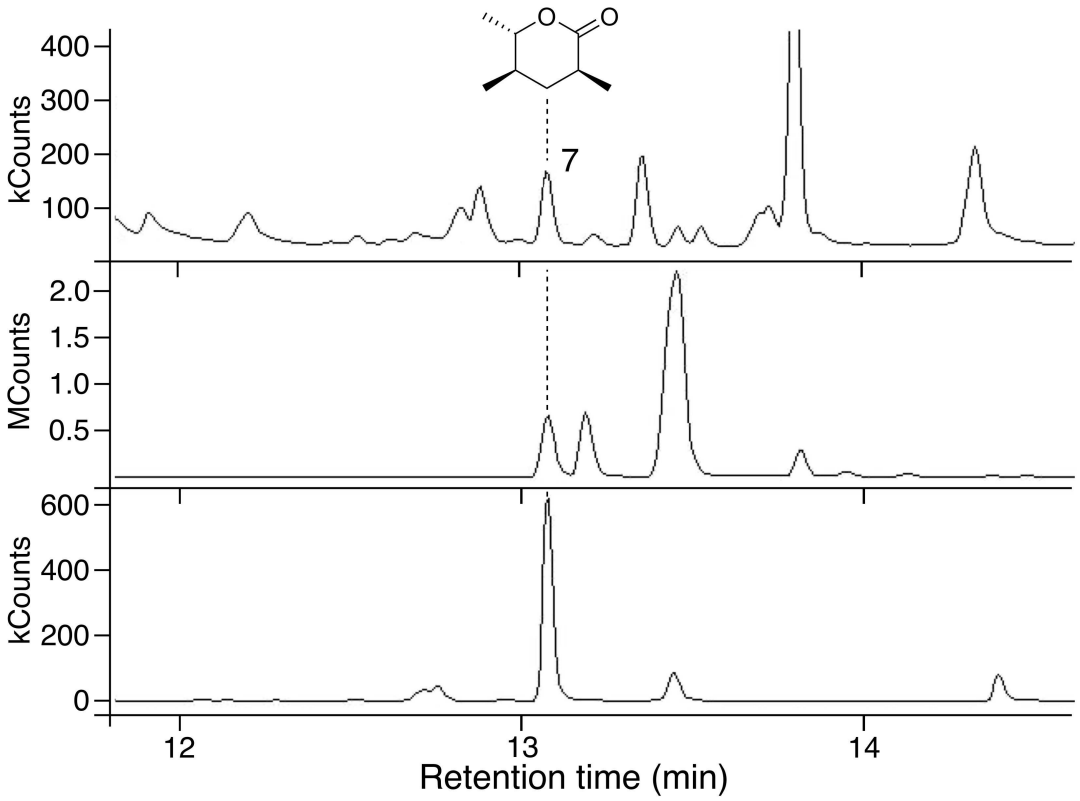

Figure 5.2. **Total ion chromatograms of *Camponotus modoc* worker hindgut extract (top), synthetic racemic 2,4-dimethyl-5-hexanolide (middle), and synthetic (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide (bottom), each analyzed by coupled gas chromatography-mass spectrometry on a DB-5 MS column. Compound 7 in hindgut extract of worker ants, the first eluting stereoisomer in 2,4-dimethyl-5-hexanolide, and (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide had identical retention times and mass spectra. Note: six isomers in the 8-component isomer blend co-eluted in the same peak**

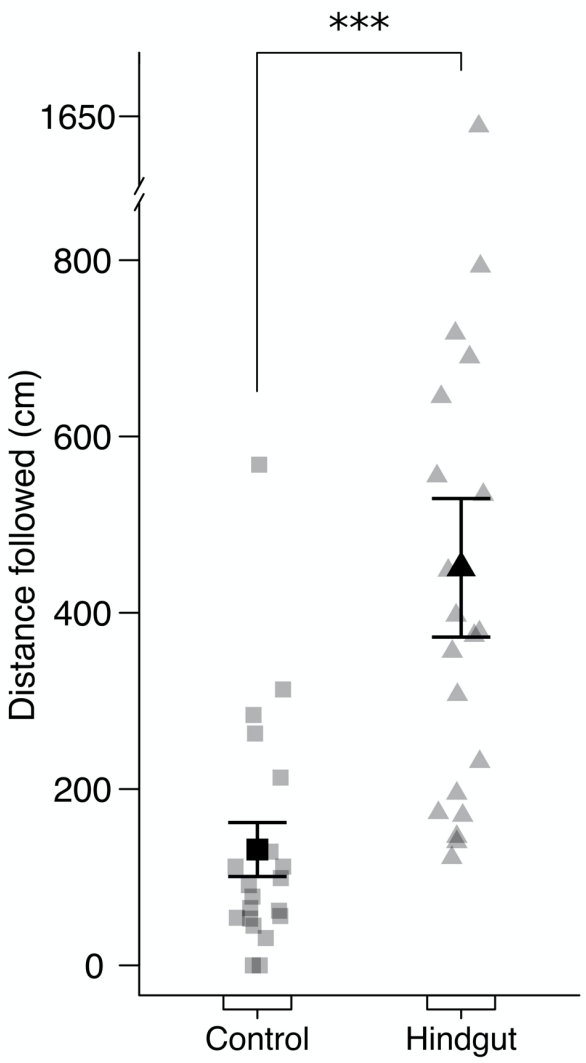

Figure 5.3. **Distances worker ants of *Camponotus modoc* followed trails of *C. modoc* hindgut extract (1 worker ant equivalent) (n = 20) or a solvent control (n = 20) applied to the circumference of a circular filter paper (diam: 185 mm) marked in 1-cm intervals (Table 5.1). Grey and black symbols show the distance that each ant and 20 ants on average (mean ± standard error), respectively, followed trails. The asterisk (*) denotes that ants followed hindgut extract trails for a longer distance than they followed solvent control trails (quasi-Poisson GLM, d.f. = 38, $t = 3.854$ $P < 0.001$). Note: re-analysis of data without the outlier (~1650 cm) in the hindgut extract treatment afforded the same statistical results.**

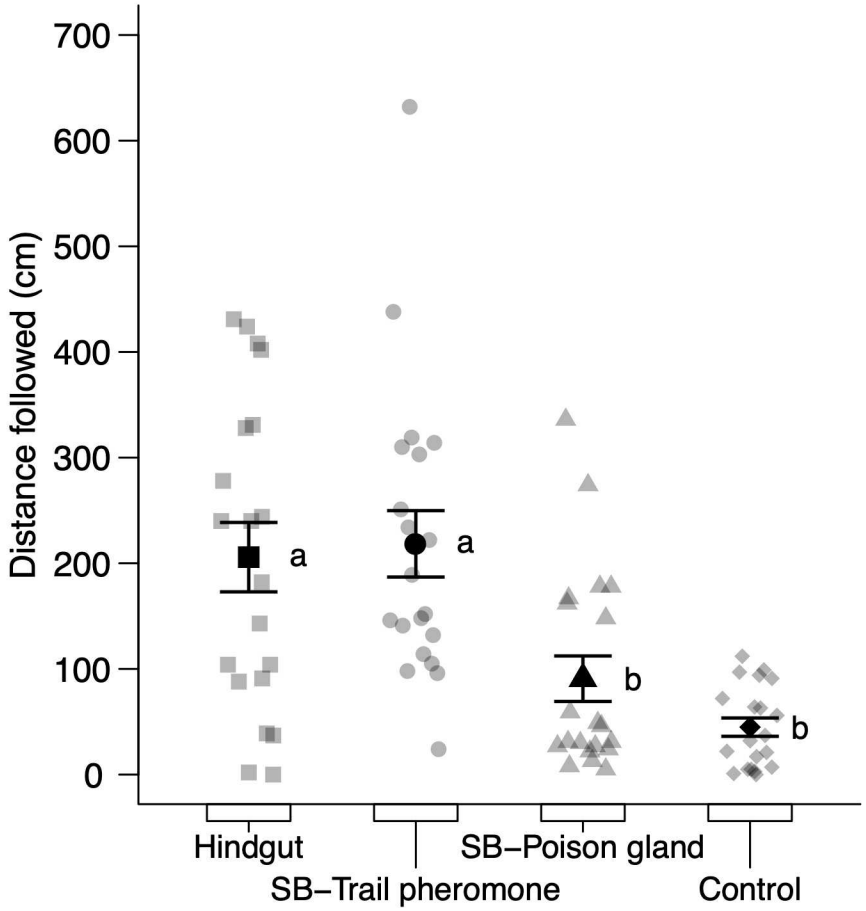

Figure 5.4. **Distances worker ants of *Camponotus modoc* followed trails of (*i*) *C. modoc* hindgut extract (1 worker ant equivalent) (n = 20); (*ii*) a synthetic blend (SB) of candidate trail pheromone components ("SB–Trail pheromone") (n = 20); (*iii*) a synthetic blend of poison gland constituents ("SB–Poison gland") (n = 20); and (*iv*) a solvent control, each trail applied to the circumference of a circular filter paper (diam: 185 mm) marked in 1-cm intervals. The detailed composition of test stimuli is reported in Table 5.1. Grey and black symbols show the distance that each ant and 20 ants on average (mean ± standard error), respectively, followed trails. The distances ants followed trails of the four test stimuli differed statistically (ANOVA, $F_{[3,76]} = 13.878$ $P < 0.0001$). Means associated with different letters are statistically different (Tukey's HSD, $P < 0.05$). Note: "SB–Trail pheromone" lacked 3,4-dihydro-8-hydroxy-3,5,7-trimethylisocoumarin which was still being synthesized.**

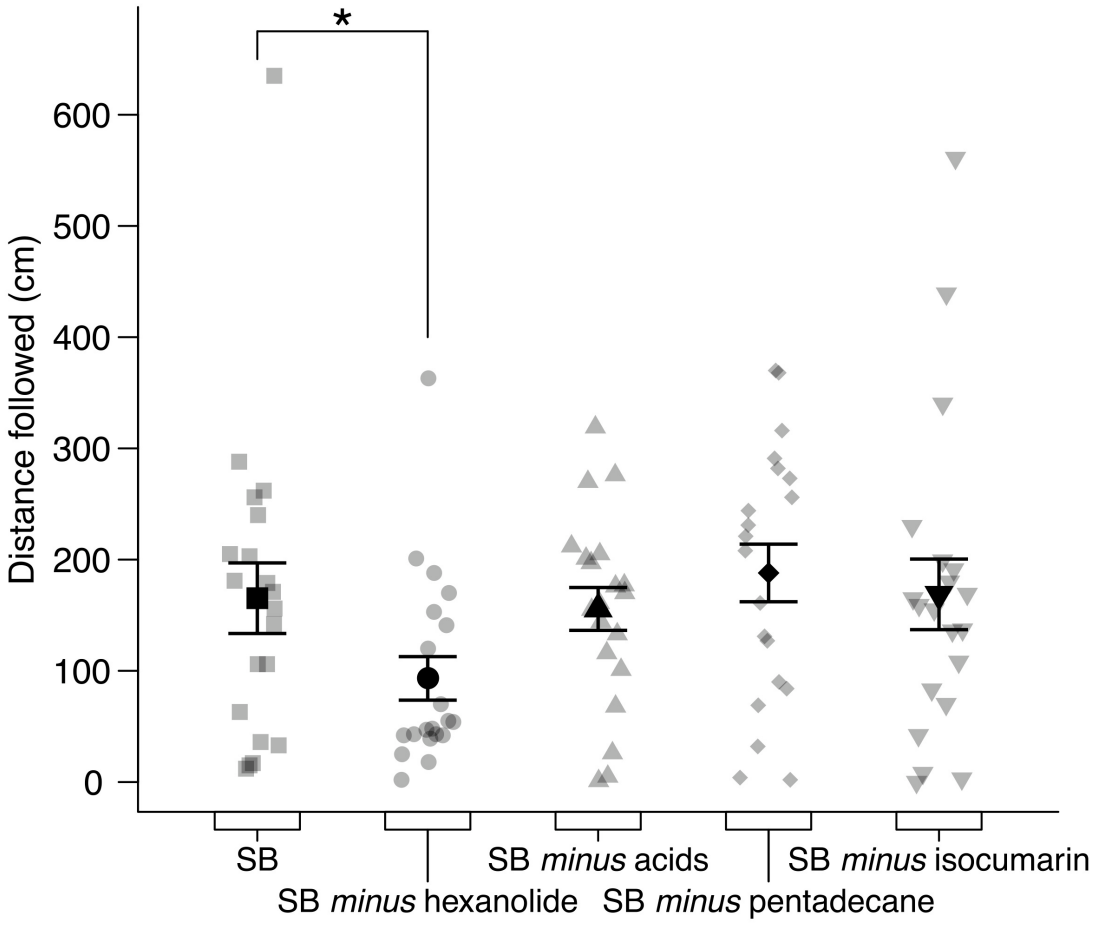

Figure 5.5. **Distances worker ants of *Camponotus modoc* followed trails of complete and partial synthetic blends (SB) of candidate trail pheromone components (n = 20 each), each trail applied to the circumference of a circular filter paper (diam: 185 mm) marked in 1-cm intervals. The composition of test stimuli is reported in Table 5.1. The compound(s) omitted from the blend are indicated by treatment. Grey and black symbols show the distance that each ant and 20 ants on average (mean ± standard error), respectively, followed trails. *A priori* contrasts between complete and partial synthetic blends revealed that the omission of 2,4-dimethyl-5-hexanolide significantly reduced the trail-following distance that ants walked (quasi-poisson GLM: d.f. = 95, t = -2.103 P = 0.038).**

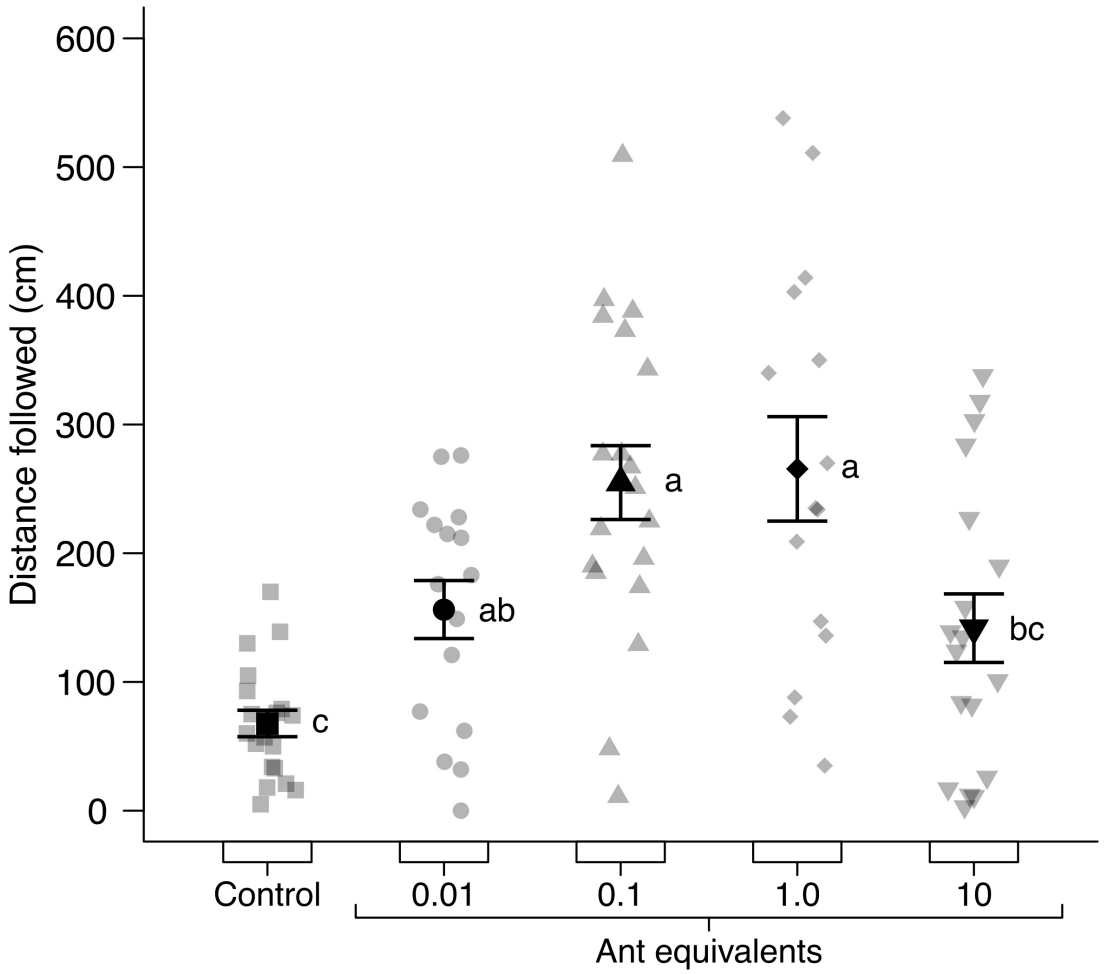

Figure 5.6. **Distances worker ants of *Camponotus modoc* followed trails of a synthetic candidate trail pheromone blend ("SB-Trail pheromone"; for blend constituents see Table 5.1) applied at different ant equivalents [0.01 (n = 16); 0.1 (n = 19); 1.0 (n = 15); 10 (n = 18); 0.00 (solvent control) (n = 19)] to the circumference of a circular filter paper (diam: 185 mm) marked in 1-cm intervals. Grey and black symbols show the distance that each ant and multiple ants on average (mean ± standard error), respectively, followed trails. The amount of trail pheromone had a significant effect on the distance ants followed a trail (ANOVA, $F_{[4, 82]} = 11.716$ $P < 0.0001$). Means associated with different letters are statistically different (Tukey's HSD, $P < 0.05$).**

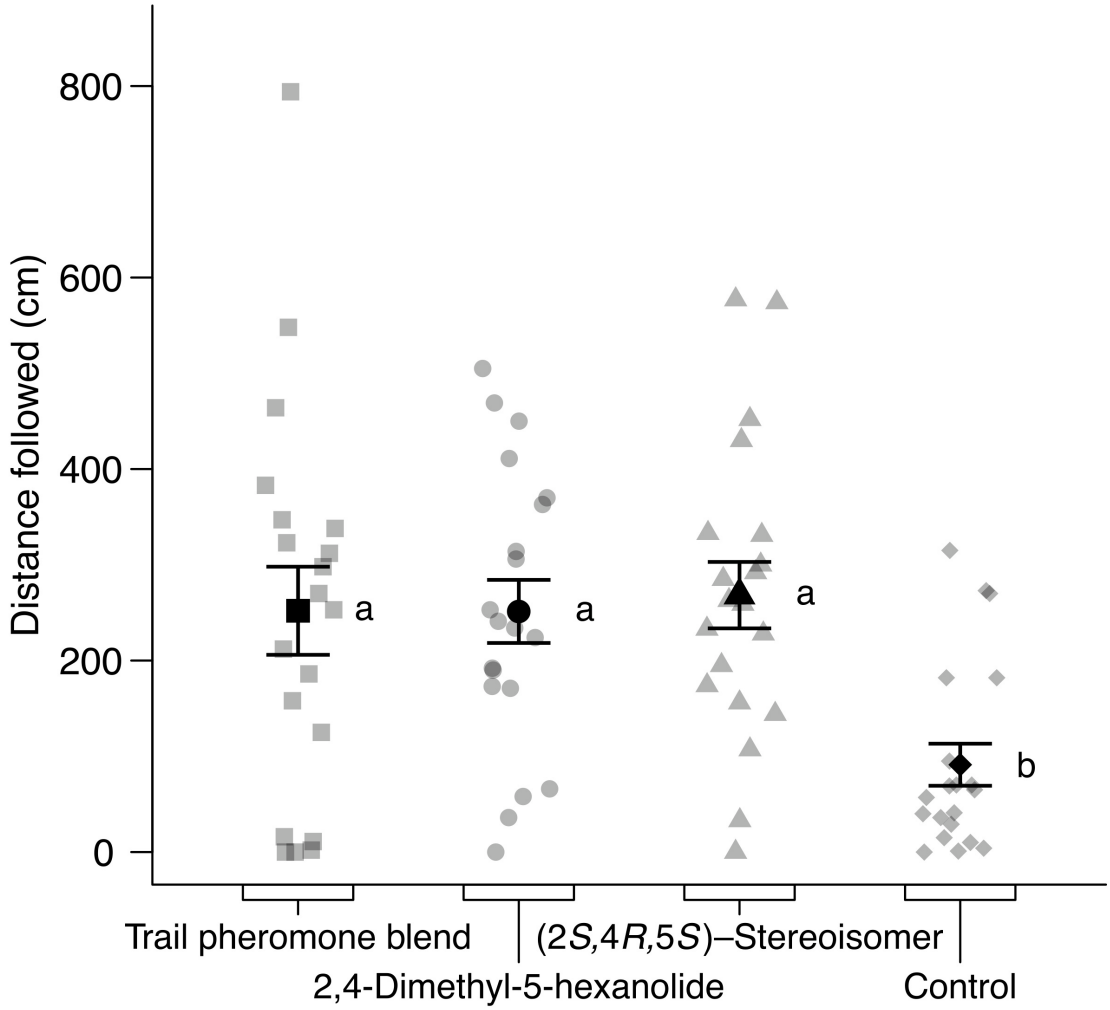

Figure 5.7. **Distances worker ants of *Camponotus modoc* followed trails of (*i*) a synthetic blend of candidate trail pheromone components ("SB-Trail pheromone"; for blend constituents see Table 5.1) (n = 20), (*ii*) 2,4-dimethyl-5-hexanolide (containing 8 stereoisomers) (32 ng), (*iii*) (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide (4 ng), and (*iv*) a solvent control. The amount of the (2*S*,4*R*,5*S*)-stereoisomer (4 ng) was identical in all three treatment stimuli. Grey and black symbols show the distance that each ant and 20 ants on average (mean ± standard error), respectively, followed trails. The distances ants followed the four trails differed statistically (ANOVA, $F_{[3,76]} = 6.8283$ $P < 0.001$). Ants followed the solvent control trail for a significantly shorter distance than each of the three pheromone trails, which were not different statistically, as indicated by the same letter (Tukey's HSD, $P < 0.05$).

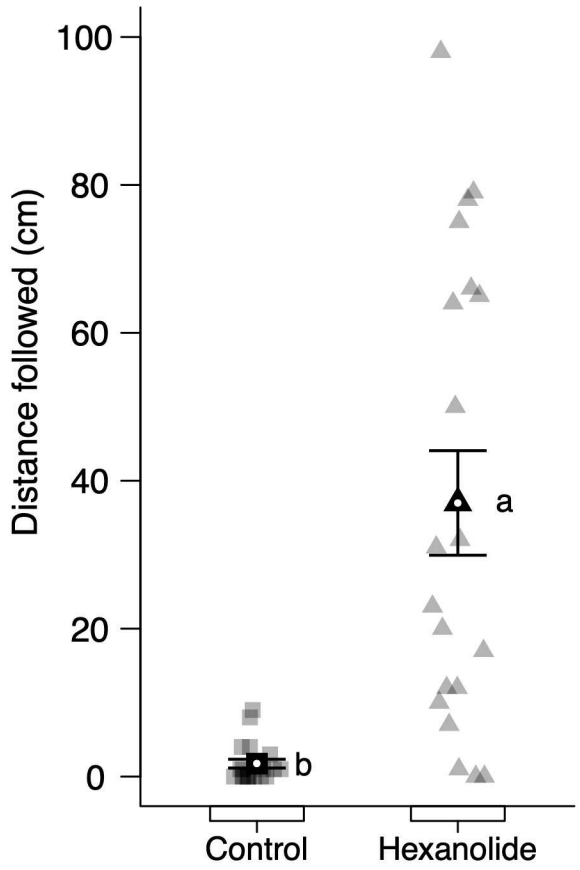

Figure 5.8. **Distances worker ants of *Camponotus modoc* followed diverging trails of (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide ("hexanolide") and a solvent control. Grey and black symbols show the distance that each ant and 20 ants on average (mean ± standard error), respectively, followed trails. Ants followed the hexanolide trail, which was tested at the same concentration (69 pg/cm) as in circular trail bioassays (Figs. 4-7), for a significantly greater distance, as indicated by different letters (Paired t-test, d.f. = 19, t = -5.0619 *P* < 0.0001).**

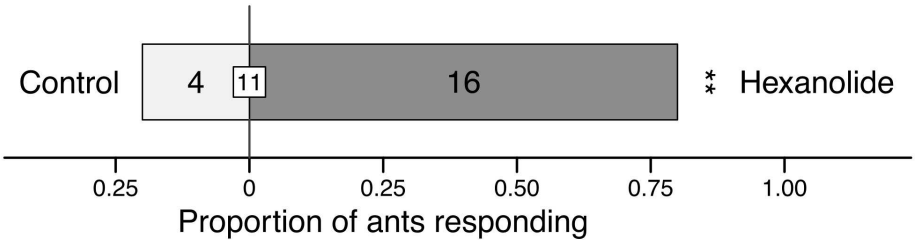

Figure 5.9 **Proportion of *Camponotus modoc* worker ants responding in binary choice Y-tube olfactometers to (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide ("hexanolide") or to a solvent control. Numbers in bars represent the number of ants selecting a test stimulus, and the number in the white inset box represents the number of non-responding ants. The asterisk (*) denotes a significant preference for the trail pheromone stimulus (Pearson's χ^2 test, d.f. = 1, χ^2 = 7.2, *P* = 0.007).**

Chapter 6.

All sugars ain't sweet: selection of particular mono-, di- and trisaccharides by western carpenter ants and European fire ants

A similar version of this chapter has been published: Renyard, A., Gries, R., Lee, J., Chalissery, J.M., Damin, S., Britton, R., and Gries, G., (2021). All sugars ain't sweet: selection of particular mono-, di- and trisaccharides by western carpenter ants and European fire ants. *R. Soc. Open Sci.* 8, 210804.

6.1. Abstract

Ants select sustained carbohydrate resources, such as aphid honeydew, based on many factors including sugar type, volume, and concentration. We tested the hypotheses (H1–H3) that Western carpenter ants, *Camponotus modoc,* seek honeydew excretions from *Cinara splendens* aphids based solely on the presence of sugar constituents (H1), prefer sugar solutions containing aphid-specific sugars (H2), and preferentially seek sugar solutions with higher sugar content (H3). We further tested the hypothesis (H4) that workers of both *C. modoc* and European fire ants, *Myrmica rubra*, selectively consume particular mono-, di-, and trisaccharides. In choice bioassays with entire ant colonies, sugar constituents in honeydew (but not aphid-specific sugar) as well as sugar concentration affected foraging decisions by *C. modoc*. Both *C. modoc* and *M. rubra* foragers preferred fructose to other monosaccharides (xylose, glucose) and sucrose to other disaccharides (maltose, melibiose, trehalose). Conversely, when offered a choice between the aphid-specific trisaccharides raffinose and melezitose, *C. modoc* and *M. rubra* favored raffinose and melezitose, respectively. Testing the favorite mono-, di- and trisaccharide head-to-head, both ant species favoured sucrose. While both sugar type and sugar concentration are the ultimate cause for consumption by foraging ants, strong recruitment of nestmates to superior sources is likely the major proximate cause.

6.2. Introduction

Adequate nutrition is vital for development, growth, functioning, and reproduction in ants [1–5]. Foraging ants assess the nutritional quality of foods, and select those that

optimize their colonies' nutritional intake and reproductive fitness [6]. In some species, foraging ants also deposit trail pheromone and engage in various behaviours to recruit nestmates, resulting in colony-level selection of profitable food sources [7,8]. Adult worker ants require primarily carbohydrates as energy sources, whereas queens and larvae also need proteins for egg production and growth, respectively [9–11]. Balancing the intake of proteins and carbohydrates is essential for the longevity of ant colonies. In all ant species studied thus far, colonies provisioned with a high carbohydrate/low protein diet lived longer than colonies provisioned with a low carbohydrate/high protein diet [12–15], implying that ants prioritise sustained carbohydrate supplies [6].

Aphid honeydew is consumed by many ant species [16] and often represents a large portion of their diet [e.g., 17]. Aphid honeydew contains mainly carbohydrates but also some amino acids, lipids, and various micronutrients [1]. Feeding aphids imbibe sugary plant sap, metabolizing mainly its amino acids, and excreting honeydew as a sugary "waste" from their anus, where ants collect it [1]. In exchange for these sugary "treats", ants protect aphids from predators and parasitoids while also providing hygienic services [16]. Although nearly all aphid species produce honeydew and would benefit from protection by "their" ant community members, only 40% of aphid species are ant-tended [16,18,19]. Aphid-ant relationships are considered unstable and dependent upon numerous ecological, physiological, and evolutionary factors [20–23]. Ants accrue benefits from tending aphids for honeydew only if its nutritional value exceeds the foraging costs and the benefits from eating the aphids [22].

Ants gauge aphid colonies as potential mutualistic partners based on both the quality and quantity of their honeydew [22]. These two honeydew characteristics vary in relation to aphid species [24–26], their host plant(s) [24,27,28], aphid instars [29], or even clonal lineages of aphids [30]. Ants preferentially consume aphid honeydew that is sugar-rich or produced in copious amounts [25–27,29]. Aphids not only obtain plant sugar, they themselves synthesize sugars, such as the trisaccharides melezitose and raffinose, to regulate osmolarity and prevent water loss [31,32]. These "aphid sugars" are rarely present in other carbohydrate sources such as floral or extra-floral nectar [25,26,33,34]. As aphid colonies that produce copious amounts of honeydew also produce large quantities of aphid-specific sugars [24–26], these aphid sugars then become indicative of a worthy mutualistic aphid partner. For example, black garden ants, *Lasius niger,* heavily tend those aphid species that produce large amounts of aphid

sugars, and preferentially feed on aphid sugars, particularly melezitose [25,26]. Aphid-specific melezitose and raffinose, e.g., prompted the relatively longest feeding times, strongest trail marking, and fastest return to nests by worker ants of *L. niger* [35]. However, many other ant species prefer common sugars or show no particular preference for aphid-specific sugars [36–38].

Here, we studied sugar-foraging of ants in the genera *Camponotus* (carpenter ants) and *Myrmica,* using the western carpenter ant, *Camponotus modoc* (subfamily: Formicinae), and the European fire ant, *Myrmica rubra* (subfamily: Myrmicinae), as model species. We selected these genera because of their species richness, contrasting life history traits (e.g., degree of aggressiveness and invasiveness), and limited knowledge of their sugar preferences. *Camponotus* spp. are taxonomically diverse and present throughout the globe [39–41], whereas *Myrmica* ants are found primarily in the Holarctic [42]. Many species of both genera consume honeydew [43–50] but little is known as to how foragers assess honeydew resources. *Camponotus* spp. in an Australian tropical rain forest preferred common sucrose to aphid-specific melezitose [36], and *C. pennsylvanicus* in North America prefer sucrose to fructose, glucose, and trehalose, but aphid-specific sugars were not tested [51].

The western carpenter ant, *Camponotus modoc* (subfamily: Formicinae)*,* is a common wood-dwelling ant in forests along the west coast of North America [52]. Workers forage up to 200 m away from their nest, using both pheromone trails and visual cues for orientation [53–55]. Foragers regularly tend to colonies of conifer aphids, *Cinara spp.,* and defend them against predators [48,56]. Foraging ants favor colonies of *Cinara curvipes* over those of *C. occidentalis* but the underlying mechanisms were not investigated [48].

The European fire ant, *Myrmica rubra* (subfamily: Myrmicinae), is an aggressive soil dwelling ant native to Europe and Central Asia [57]. Inadvertently introduced to the East and West Coasts of North America, *M. rubra* dwells in habitats such as lawns, forests, and urban settings [57–59]. *Myrmica rubra* strongly competes for food resources and is aggressive towards and displaces native ants, including *C. modoc* [57,60]. Workers forage within 2 m of a nest entrance (Higgins, pers. comm.) and tend to various hemipterans, including aphids [44–47,60]. In their native range, *M. rubra* uses fructose for short-term energy and glucose for both direct or stored energy, whereas galactose

units in di- or tri-saccharides reduce feeding [61]. Worker ants of *M. rubra* sense sucrose, maltose, raffinose and melezitose at a lower concentration than glucose and fructose [61]. As yet, no study has tested entire *M. rubra* colonies with queens and brood for their sugar preferences when offered choices between multiple sugars.

Here, we tested the hypotheses (H1–H3) that *C. modoc* colonies seek aphid honeydew based solely on the presence of sugar constituents (H1), prefer sugar solutions containing aphid-derived sugars (H2), and preferentially seek sugar solutions with higher sugar content (H3). We further tested the hypothesis (H4) that *C. modoc* and *M. rubra* distinguish between, and selectively seek, particular mono-, di- and tri-saccharides.

6.3. Materials and methods

6.3.1. Ants and aphids

We reared *C. modoc* as previously detailed [55]. Briefly, we excised *C. modoc* nests (three in 2016, one in 2017, and two in 2018) from forest logs and maintained them in an outdoor undercover area of the Science Research Annex (49°16′33″ N, 122°54′55″ W) on the Burnaby campus of Simon Fraser University, where ants experienced natural cycles of light and temperature throughout the year. We housed ant-infested log sections in large plastic bins connected via polyvinylchloride (Nalgene™) tubing to glass tanks (41 × 21 × 26 cm) which served as the ants' foraging area which was provisioned with insect prey, honey, apples, canned chicken, and 20% sugar (sucrose) water ad libitum.

We collected and reared *M. rubra* drawing on a previous report [62] but modifying the procedure. In the summer of 2019, we excavated six nests of *M. rubra* at the Inter River Park (North Vancouver, BC, Canada). We placed these nests with 'their' soil in separate glass aquaria (26 × 21 × 40.6 cm; 30.5 × 26 × 50.8 cm) or large totes (58 × 43 × 31 cm) with the above-soil space serving as the nests' foraging area. Nests were kept indoors in the Science Research Annex (see above) at 25 °C and a photoperiod of 12 h L to 12 h D. We sprayed nests with water and provisioned them with food (apples, insect prey) 2-times per week, replacing test tubes (10-40 mL) with water reservoirs as needed.

We obtained conifer aphids, *Cinara splendens*, from a local nursery by purchasing a potted 2.4-m tall Douglas-fir tree, *Pseudotsuga menziesii*, infested with multiple *C. splendens* colonies tended by *C. modoc*. We planted the tree near the Science Research Annex and enclosed three of its aphid-infested branches with mesh bags to exclude foraging ants, predators, and parasitoids. Aphid taxonomic identity was confirmed by Eric Maw at the Canadian National Collection (species reference # 2019-107).

6.3.2. Honeydew collection

To collect honeydew (every one or two days), we removed the mesh bag from aphid-infested branches, and then scooped and scraped any honeydew present on needles near aphid colonies using a 5-µL microcapillary tube. This unusual collection procedure took into account that the honeydew was too viscous to enter the tube via capillary action. To remove the honeydew from the capillary tube for chemical analyses, we stirred the tube in a 3-mL vial ("vial 1") containing distilled water (1 mL), and filtered the resulting watery honeydew through glass wool into another vial ("vial 2") with a known tare weight. We then re-rinsed vial 1 with 0.5 mL of distilled water, and decanted and filtered this rinse also into vial 2. Following gentle water evaporation from vial 2 at 35 °C, we allowed vial 2 to cool to room temperature, and then determined the weight of the honeydew residue [containing sugars, amino acids, lipids, various micronutrients, and possibly even some needle surface chemicals (see honeydew collection method)] by subtracting the tare weight of vial 2 from the total weight. We placed the capped vial in a -4 °C freezer, and continued honeydew collections for a total of three samples.

6.3.3. Analytical chemistry

We dissolved 50 mg of dry honeydew (see above) in a mix of water and acetonitrile (ACN) (1 mL; 1:1), evaporated a 100-µL aliquot of the mix to dryness, and converted the honeydew sugars to trimethylsilyl (TMS) derivatives for GC-MS analyses. To this end, we treated the honeydew residue with a solution of pyridine (10 µl) and bis(trimethylsilyl)trifluoroacetamide [BSTFA (25 µl)] containing 1% of trimethylchlorosilane (TMCS); Sigma-Aldrich, St. Louis, MO 63103, USA), and kept the reaction mixture 3 h at 70 °C [63]. After evaporating the mixture to dryness, we added

pentane and hexane (1 mL; 1:1) and injected a 1-µl aliquot into an Agilent 7890B MSD (Agilent Technologies Inc., Santa Clara, CA 95051, USA) interfaced with a gas chromatograph (GC - 5977A) fitted with DB-5MS column (30 m × 0.25 mm ID; film thickness: 0.25 µm) (Agilent). One of two GC oven programs was used: (1) 100 °C (1 min), 20 °C · min-1 to 300 °C (held 60 min); (2) 100 °C (1 min), 10 °C · min-1 to 240 °C, 25 °C · min-1 to 300 °C (held 20 min). The second GC oven program was run to help separate the mono- and disaccharides in the analyte. The injector port was set to 280 °C and the transfer line to 300 °C.

With our research hypotheses in mind, sugar analyses focussed on those D-form ring sugars that are commonly found in aphid honeydew. We prepared 10-mg samples of each commercially available sugar (Table C1), correcting the weight of hydrated sugars (maltose, trehalose, raffinose, melezitose) according to hydration levels. We BSTFA-treated each sugar separately (see above), prepared distilled-water solutions of the BSTFA derivatives at three concentrations (100 ng/µL, 10 ng/µL, 1 ng/µL), and analyzed aliquots of each sample by GC-MS.

We identified and quantified (derivatized) sugars in aphid honeydew by comparing their mass spectra, retention times, and ion counts with those of authentic sugar standards. To assign a molecular structure to an unknown trisaccharide, we isolated it for NMR analysis by high performance liquid chromatography (HPLC) (Waters HPLC system; 600 Controller, 2487 Dual Absorbance Detector, Delta 600 pump; Waters Corp., Milford, MA 01757, USA), eluting analytes on an apHera NH2 Polymer column (250 mm × 4.6 mm, 5 µm particle size; Advanced Separation Technologies Inc., Whippany, NJ 07981, USA) with an isocratic flow (1 mL min-1) of ACN and H2O (3:1). To approximate the elution time of the unknown tri-saccharide in aphid honeydew for collection, we determined the retention times of two authentic trisaccharides (melezitose: 17.4 min; raffinose: 19.2 min) and, based on this information, then processed the honeydew. In each of six HPLC runs, we injected a 25-µl aliquot containing approximately 6 mg of the honeydew sugars, and collected 0.5-min fractions between 16 and 20 min. To determine the fraction containing the unknown trisaccharide, we combined equivalent time fractions, evaporated aliquots (10%) of each (combined) fraction to dryness, and treated them with BSTFA for GC-MS analyses of the sugar derivatives. We then evaporated the fraction containing the unknown (ca. 350 µg total) to dryness, and dissolved it in D2O for both 1H and 13C NMR spectroscopic analysis.

NMR spectra were obtained on a Bruker Instrument (Avance 600 NMR) equipped with a QNP cryoprobe.

6.3.4. General sugar preference bioassays

Western carpenter ants

As *C. modoc* nests are generally most active on warm and sunny days (AR, pers. obs.), we ran bioassays on days with at least a mix of sun and cloud and with the atmospheric pressure rising or constant. At 07:15 on any bioassay day, we removed all food from the foraging arenas of colonies, starving ants for 4 h prior to the onset of bioassays (the maximum amount of time ants could be without food before they attempted to chew their way out of housing containers). During this time, we prepared aqueous sugar solutions (5% by weight (w/v)), and pipetted 1.0-mL aliquots of each solution into labelled plastic Eppendorf tubes (1.5 mL; Thermo Fisher Scientific, Waltham, MA 02451, USA) stuffed with a 1-cm-long piece of a cotton dental wick (Richmond Dental & Medical, Charlotte, NC 28205, USA) to facilitate food consumption by ants without spillage. Once fully prepared, we weighed tubes so that food consumption by ants and water evaporation during subsequent bioassays could be determined. For each sugar solution bioassayed, a corresponding “evaporation control” Eppendorf was taped to the lid underside of the bioassay arena (Fig. 6.1A) inaccessible to ants. Tubes remained capped prior to the onset of bioassays. All experiments on carpenter ants were conducted during the summer of 2018.

As ant colonies make resource foraging decisions collectively [6], and form long-term associations with aphid colonies [e.g., 48], we tested entire colonies of *C. modoc* and *M. rubra* and measured their collective consumption of sugar solutions over the course of several hours. The number of *C. modoc* colonies (n = 6) we tested in experiments was limited by the number of nests that we could locate in (mountainous) forests, and by the size and weight of ant-infested log sections that we could haul out of forests and house in large bins (64 × 79 × 117 cm) in an outdoor enclosure of the Science Research Annex. We used corresponding numbers of *M. rubra* colonies in comparative experiments. The numbers of ant colonies we tested in our study correlate with those reported in related studies [e.g., 25].

We tested consumption of sugar solutions by individual ant colonies in large Plexiglass bioassay arenas (50.5 × 30.5 × 33 cm), the upper inner walls of which coated with a 50/50 mix of Petroleum jelly (Unilever, London, England) and white Paraffin oil (Anachemia, Lachine, QC H8R1A3, CA) to prevent ants from escaping, and the top of which covered with a mesh lid to facilitate ventilation. In each arena, we presented ants with a choice of 2–4 Eppendorf tubes each containing a different sugar solution or a plain water control. We taped tubes to the arena floor 22 cm away from the entrance hole of the arena and spaced them equidistantly in an arc (Fig. 6.1A), with tube positions randomly assigned in each replicate. Just prior to the onset of bioassays, we opened all Eppendorf tubes (including the evaporation controls), and connected individual bins housing an ant nest to a bioassay arena via Tygon® tubing (diam.: 2.54 cm) and barbed plumbing connectors (diam.: 2.54 cm), thereby allowing ants to enter and exit the bioassay arena on their own accord. After ants had foraged for 165 min, we capped and weighed all tubes to obtain consumption rates (amount of sugar solution consumed during 165 min), wiped bioassay arenas with hexane and ethanol (70%), and washed plumbing fixtures and Tygon® tubing with warm soapy water followed by a water rinse.

European fire ants

Foraging activity of *M. rubra* was not noticeably affected by weather (JMC, pers. obs.), allowing us to run bioassays on any day. We deprived *M. rubra* nests of food and water for 24 h and at least 2 h, respectively, prior to the onset of bioassays. As we had prepared Eppendorf tubes with aqueous sugar solutions, or with plain water (control stimulus), well before bioassays, we kept tubes frozen and thawed them 2 h before bioassays. For each bioassay replicate, we taped the Eppendorf tubes horizontally and equidistantly along the perimeter of a jar lid (diam: 15 cm), randomizing the position of tubes and the direction of their opening (Fig. 6.1B). We then placed this lid at the centre of the ant's 'nesting' tank and taped the corresponding evaporation control Eppendorf tubes on the underside of the tank lid inaccessible to ants (Fig. 6.1B). To initiate a bioassay, we uncapped all tubes and allowed ants to forage. As worker ants of *M. rubra* are significantly smaller and 12-times lighter than those of *C. modoc* [64], and accordingly consume less sugar solution per unit time, we extended the total bioassay time from 165 min (as in bioassays with *C. modoc*) to 360 min. To terminate a bioassay, we capped and weighed tubes.

6.3.5. Specific experiments

H1: Worker ants of C. modoc seek aphid honeydew based solely on the presence of sugar constituents (Exp. 1)

To test H1, we bioassayed aqueous solutions of aphid honeydew versus a synthetic blend of sugars identified in honeydew. To prepare honeydew test stimuli, we collected aphid honeydew once on each of five separate days (25 and 30 July, 4, 9 and 20 August 2018) into five separate (labelled) vials with known tare weight containing 1 mL of distilled water. After evaporating each sample to dryness, we re-weighed each vial to obtain the weight of the residual honeydew sugars and other constituents. We stored vials in a -4 °C freezer until ready for testing in bioassays.

To bioassay honeydew/sugar consumption by ant workers, we prepared 12 mL of an aqueous honeydew solution, 6 × 1 mL for bioassaying consumption by six ant nests and 6 × 1 mL to serve as corresponding evaporation controls. To prepare the 12-mL aqueous honeydew, we reconstituted the dry honeydew in each of the five vials (see above) by adding distilled water (1.2 mL) to each vial and shaking it until the honeydew was fully dissolved. We then decanted the five honeydew solutions into a single vial, re-rinsed each vial with an additional 1.2 mL of water, and combined all rinses in a single vial for a total volume of 12 mL. We shook the combined solution and then placed it in a -4 °C freezer to be tested in bioassays later. The combined solution had a sugar content of 4.5% (w/v).

We prepared a synthetic blend (SB) of sugars resembling the quantity and ratio of specific sugar constituents in aphid honeydew [fructose (14.3%), glucose (14.3%), sucrose (28.6%), trehalose (28.6%), raffinose (14.3%); see Fig. 6.2)]. We prepared 12 mL of the SB with a total concentration of these sugars [4.5% (w/v)] resembling that in reconstituted honeydew (see above), and stored the solution at -4 °C. Here and in experiments below, we tested low sugar solutions (4.5–5%), knowing that ants can distinguish between types of sugar at only 2.5% (data not shown), and anticipating better discrimination between sugar types at low concentration.

In each of six replicates, we offered a colony a choice between aqueous honeydew (1 mL) and aqueous SB (1 mL).

H2: Worker ants of C. modoc prefer sugar solutions containing aphid-derived sugars (Exp. 2)

To test H2, we bioassayed the complete aqueous SB (see H1) versus a partial aqueous SB lacking the aphid-derived sugar raffinose, adjusting the total sugar concentration in both the complete and the partial SB to the same level [5% (w/v)].

In each of five replicates, we offered a *C. modoc* colony a choice between the complete aqueous SB (1 mL) and the partial aqueous SB (1 mL).

H3: Worker ants of C. modoc preferentially seek sugar solutions with higher sugar content (Exp. 3)

To test H3, we offered each of four *C. modoc* colonies aqueous solutions of fructose (a preferred monosaccharide; see Results) with increasing fructose content [5%, 20%, 40%, or 70% (w/v)].

H4: Worker ants of C. modoc and M. rubra distinguish between, and selectively seek, particular mono-, di- and tri-saccharides (Exps. 4-7)

To test H4, we offered six *C. modoc* colonies and six *M. rubra* colonies choices between aqueous solutions [5% (w/v)] of (i) single monosaccharides [D-(+)-xylose, D-(-)-fructose, or D-(+)-glucose] (Exps. 4A, B), (ii) single disaccharides [D-(+)-sucrose, D-(+)-maltose monohydrate, D-(+)-trehalose dihydrate, or D-(+)-melibiose] (Exps. 5A, B), (iii) single trisaccharides [D-(+)-raffinose pentahydrate or D-(+)-melezitose] (Exps. 6A, B), and (iv) the preferred monosaccharide [D-(-)-fructose], disaccharide [D-(+)-sucrose], and trisaccharide [D-(+)- raffinose pentahydrate and D-(+)-melezitose, respectively] (Exp. 7A, B) (see Results). All bioassays with *M. rubra* (Exps. 4B, 5B, 6B, 7B), but not with *C. modoc* (Exps. 4A, 5A, 6A, 7A), included plain water (1 mL) as an additional test stimulus.

6.3.6. Statistical analyses

We analyzed data using R (V3.5.1) and R-studio (V1.1.456) [65]. To calculate the amount of each sugar test solution that was consumed by a colony, we first determined the weight loss of the corresponding evaporation control solution, and then subtracted this value from the weight loss of the test solution. To account for differences in colony size and foraging activity between colonies, we analyzed proportions, rather than

absolute amounts, of sugar solutions consumed. To obtain proportional consumption data for a colony in any experimental replicate, we divided the amount (weight) of each sugar solution consumed by the total amount of sugar solution consumed. As parametric methods have greater statistical power than non-parametric methods, and as we wanted to compare mean consumption data of sugar solutions (rather than ranks assigned to consumption data [66]), we analyzed proportional consumption data for each experiment using a linear mixed effects model [67], with sugar solution as a fixed effect and ant colony as a random effect (to account for simultaneous choices by ants between sugar solutions). As colonies of *M. rubra* did not consume certain sugar solutions, some 'consumption' data became < 0 following weight loss subtraction due to passive water evaporation measured in evaporation controls (see above). To improve model fit, we excluded from analyses those sugar solutions which (in one sample T-tests) had mean 'consumption' values (in grams) significantly less than 0. Following this procedure, sugar solutions with remaining < 0 consumption values were assigned '0' values (< 0 consumption is not possible), and 0-value data together with all other data were entered into the statistical model. We used a likelihood ratio test to compare the effect of sugar treatment on the mean proportion of sugar solutions consumed by colonies. We compared the pairwise differences in the estimated marginal mean proportion of sugar solution consumed between treatments with a Tukey's HSD test using the emmeans package which is appropriate for linear-mixed effects models (emmeans package; [68]). For experiment 3, visual inspection of data and comparison of Akaike Information Criterion (AIC) values revealed that the natural log of the percent-fructose treatment offered the best fit for the proportion of sugar solution consumed by ants. We used a likelihood ratio test to compare the effect of increasing percent fructose solution in our model versus an intercept-only model. For all experiments, we assessed model fit using a Q-Q plot and a residuals vs fitted plot. We generated graphics in R-studio and Inkscape (V1.0.2).

6.4. Results

6.4.1. Sugar constituents in *C. splendens* honeydew

GC-MS analyses of *C. splendens* honeydew (1-µL aliquots containing ca. 25 µg of total constituents) revealed the presence of two monosaccharides [D-(-)-fructose (50

µg of total 50-mg sample), D-(+)-glucose (50 µg)], three disaccharides [D-(+)-sucrose (100 µg), D-(+)-turanose (10 µg), D-(+)-trehalose (50 µg)] and one trisaccharide [D-(+)-raffinose pentahydrate (50 µg)] (Fig. 6.2). Erlose as a second trisaccharide was identified by NMR spectroscopy.

6.4.2. H1: Worker ants of *C. modoc* seek aphid honeydew based solely on the presence of sugar constituents (Exp. 1)

A honeydew solution of *C. splendens* and a blend of select synthetic honeydew sugars tested at equal concentration prompted similar consumption rates by *C. modoc* (likelihood ratio test, $\chi^2 = 0.0196$, DF = 1, P = 0.89; Fig. 6.3A), indicating that honeydew constituents (e.g., amino acids) other than these select sugars did not modulate foraging responses.

6.4.3. H2: Worker ants of *C. modoc* prefer sugar solutions containing aphid-derived sugars (Exp. 2)

Two solutions of synthetic sugars, tested at equal concentration with or without raffinose (an aphid-derived saccharide), elicited similar consumption rates by *C. modoc* (likelihood ratio test, $\chi^2 = 2.521$, DF = 1, P = 0.11; Fig. 6.3B), indicating that the presence of raffinose did not increase foraging responses.

6.4.4. H3: Worker ants of *C. modoc* preferentially seek sugar solutions with higher sugar content (Exp. 3)

When offered aqueous fructose solutions with increasing fructose content [5%, 20%, 40%, or and 70% (w/v)], *C. modoc* preferentially consumed solutions with higher fructose content (likelihood ratio test, $\chi^2 = 14.152$, DF = 1, $P < 0.001$; Fig. 6.4).

6.4.5. H4: Worker ants of *C. modoc* and *M. rubra* distinguish between, and selectively seek, particular mono-, di- and tri-saccharides (Exps. 4-7)

Experiment 4: Choices between monosaccharides

Solutions of single monosaccharides differed in their ability to prompt consumption by *C. modoc* (likelihood ratio test, $\chi^2 = 7.7015$, DF = 2, P = 0.02; Fig. 6.5A)

and by *M. rubra* (likelihood ratio test, χ^2 = 71.547, DF = 3, P < 0.00001; Fig. 6.5B). However, we did not detect differences in consumption by colonies of *C. modoc* in post-hoc pairwise comparisons between any of the sugar solutions (Tukey HSD: fructose *vs* glucose: T = 2.267, P = 0.11; fructose *vs* xylose: T = 2.601, P = 0.06; glucose *vs* xylose: T = 0.333, P = 0.94). Numerically, fructose had higher consumption rates than the other monosaccharides, but this difference could not be shown statistically due to the limited sample size. Colonies of *M. rubra* consumed more of the fructose solution than of the glucose or xylose solution, with the xylose solution and plain water prompting equally low consumption (Tukey HSD: fructose *vs* glucose: T = 8.071, P < 0.0001; fructose *vs* xylose: T = 16.267, P < 0.0001; fructose *vs* water: T = 16.660, P < 0.0001; glucose *vs* xylose: T = 8.196, P < 0.0001; glucose *vs* water: T = 8.588, P < 0.0001; xylose *vs* water: T = -0.392, P = 0.9787)

Experiment 5: Choices between disaccharides

Solutions of single disaccharides differed in their ability to prompt consumption by *C. modoc* (likelihood ratio test, χ^2 = 15.239, DF = 3, P = 0.0016; Fig. 6.6A) and *M. rubra* (likelihood ratio test, χ^2 = 55.82, DF = 1, P < 0.00001; Fig. 6.6B). Colonies of *C. modoc* consumed more of the sucrose solution than of maltose, melibiose or trehalose solutions (Tukey HSD: sucrose *vs* maltose: T = -3.546, P = 0.02; sucrose *vs* melibiose: T = -3.633, P = 0.02; sucrose *vs* trehalose: T = 3.246, P = 0.03), with the latter three solutions prompting similarly low and equal consumptions (maltose *vs* melibiose: T = 0.088, P = 0.99; maltose *vs* trehalose: T = -0.300, P = 0.99; melibiose *vs* trehalose: T = -0.388, P = 0.98). As consumptions of melibiose solutions, trehalose solutions and of water by *M. rubra* colonies did not differ significantly from zero (Table C2), we compared proportional consumption only between maltose and sucrose solutions, with the latter being preferred (Tukey HSD: T = 32.212, P < 0.0001).

Experiment 6: Choices between trisaccharides

Solutions of single trisaccharides differed in their ability to prompt consumption by *C. modoc* (likelihood ratio test χ^2 = 17.498, DF = 1, P < 0.0001; Fig. 6.7A) and *M. rubra* (likelihood ratio test χ^2 = 53.949, DF = 2, P < 0.00001; Fig. 6.7B). Colonies of *C. modoc* consumed more of the raffinose solution than of the melezitose solution (Tukey HSD: melezitose *vs* raffinose: T = -5.743, P = 0.002). In contrast, colonies of *M. rubra* consumed more of the melezitose solution than of the raffinose solution (Tukey HSD:

melezitose *vs* raffinose: T = 5.672, P < 0.0005) and more of the melezitose or raffinose solution than of a plain water control (Tukey HSD: melezitose *vs* water: T = 16.618, P < 0.0001; raffinose *vs* water: T = 10.946, P < 0.0001).

Experiment 7: Choices between most preferred mono-, di- and trisaccharides

When concurrently-offered, single-sugar solutions of the mono-, di-, or trisaccharide preferentially consumed by *C. modoc* and *M. rubra* in preceding experiments 4–6, prompted similar consumption by *C. modoc* (likelihood ratio test, χ^2 = 2.0904, DF = 2, P = 0.3516; Fig. 6.8A) but dissimilar consumption by *M. rubra* (likelihood ratio test, χ^2 = 50.176, DF = 3, P < 0.00001; Fig. 6.8B). Colonies of *M. rubra* consumed more of the sucrose than of the fructose solution (Tukey HSD: T =3.321, P = 0.0216), as much fructose as melezitose solution (T = -2.154, P = 0.1813), and as much sucrose as melezitose solution (T = -1.167, P = 0.6557). Any sugar solution prompted more consumption than plain water (fructose *vs* water: T = 7.501, P < 0.0001; melezitose *vs* water: T = 9.655, P < 0.001; sucrose *vs* water: T = 10.822, P < 0.0001).

6.5. Discussion

As predicted, *C. modoc* sought honeydew based solely on the presence of sugar constituents (Fig. 6.3A) and preferentially consumed sugar solutions with higher sugar content (Fig. 6.4). Also as predicted, *C. modoc* and *M. rubra* distinguished between, and selectively sought, particular mono-, di- and trisaccharides (Figs. 6.5–8). Unexpectedly, however, aphid-derived sugar did not affect sugar foraging decisions by *C. modoc* (Fig. 6.3B). Below, we shall elaborate on our results.

Equal consumption by *C. modoc* workers of *C. splendens* honeydew (containing fructose, glucose, sucrose, turanose, trehalose, raffinose, and erlose among other constituents; Fig. 6.2), and of a synthetic sugar blend containing these same sugars (except for turanose and erlose) but lacking other honeydew constituents, propounds a primary role of sugars driving the decisions of honeydew-foraging *C. modoc*. Moreover, equal consumption by *C. modoc* workers of synthetic sugar blends with or without the aphid-specific sugar raffinose indicates that aphid-specific sugars do not drive sugar-foraging decisions by *C. modoc.*

While the sugar composition of *C. splendens* honeydew – in general – resembles that of other aphids including *Cinara* spp. [24–27,69], honeydew sugar compositions can vary with aphid species and according to host plant. For example, both *C. pectinatae* and *C. confinis* feeding on white fir, *Abies alba*, produced 7.5-times more erlose than melezitose, whereas *C. pilicornis* and *C. piceae* feeding on spruce, *Picea abies*, produced much less erlose than melezitose [69], a sugar which we did not detect in our study. As common sugar constituents, fructose, glucose, and sucrose occur not only in honeydew but in many other carbohydrate sources including floral and extra-floral nectar [25,33,70]. In contrast, oligosaccharides like melezitose are biosynthesized by aphids [31,32] and thus are “signature” sugars of aphid honeydew [but see 70].

The effect of aphid signature sugars on foraging responses by ants is not consistent among the ant species studied thus far. For example, the presence and absence of raffinose in sugar blends had no effect on foraging responses by *C. modoc* in our study (Fig. 6.3B). Similarly, many other ant species preferred common sugars to aphid-derived sugars or had no preference [36–38,72], whereas *L. niger* and the red imported fire ant, *Solenopsis invicta*, preferred aphid-derived sugars (melezitose, raffinose) to the common sugar sucrose [25,73,74]. Considering that ants often consume honeydew as a carbohydrate source [16], it seems perplexing that aphid-derived sugars are not a universal feeding stimulant [36–38,72]. However, depending on the ants’ foraging ecology, cues other than sugar type may inform foraging responses. For example, worker ants of *L. niger* recognize sugar-valuable aphid colonies based on their cuticular hydrocarbon profile [75] and they visit clonal lineages of black bean aphids, *Aphis fabae*, irrespective of low or high melezitose content in honeydew secretions [30,76]. Decisions by honeydew-foraging ants are further affected by aphid colony size [77–79], the volume and sugar concentration of honeydew [27,29,36,80], and the distance of sugar resources to the nest of foraging ants [81,82].

Worker ants of *C. modoc* and *M. rubra* clearly distinguished between different types of sugar. When offered a choice between separate solutions of monosaccharides (glucose, fructose, xylose), both *C. modoc* and *M. rubra* preferentially consumed fructose solutions (Fig. 6.5A,B). Their selection of a specific disaccharide was equally consistent. When offered a choice between separate solutions of maltose, melibiose, trehalose or sucrose, workers of both *C. modoc* and *M. rubra* preferentially consumed the sucrose solution (Fig. 6.6). As ants and bees dislike unexpected flavours [83,84],

and as both *C. modoc* and *M. rubra* may have been used to the sucrose taste in their rearing diet, it is conceivable – but not very likely – that the sucrose preference of ants in our study was affected by the rearing diet. Irrespectively, the sucrose preference revealed in our study confirms findings in related studies with other species of ants [36–38,72].

The choice of aphid-specific trisaccharides differed between *C. modoc* and *M. rubra*. Workers of *C. modoc* consumed more raffinose than melezitose, whereas *M. rubra* workers favored raffinose over melezitose (Fig. 6.7). When offered a choice then between the specific mono-, di- or trisaccharides that were favored in preceding bioassays, *C. modoc* workers equally consumed solutions of fructose, raffinose or sucrose, whereas *M. rubra* workers favored sucrose and melezitose solutions to fructose solutions (Fig. 6.8), revealing equal interest in a common sugar and an aphid-derived sugar. As all sugars (except for xylose) tested in experiments 4–6 have near-identical molar mass, it is the structure and resulting taste of sugar molecules, rather than the number of molecules in water solution, that seem to guide sugar foraging decisions by ants.

The top choice of sucrose by *C. modoc* and *M. rubra* as (one of) their favorite sugars is likely linked to both its nutritional value and digestibility by these ants. Enzymes such as invertase that are capable of breaking sucrose down to its glucose and fructose constituents occur commonly in ants [85–87]. They are reported to be present in the digestive tract of several *Camponotus* species [85,86] and are likely present in the digestive tract of *M. rubra* [61]. As fructose and glucose readily cross the intestinal barrier, they can then be metabolized as energy sources [86], with fructose shown to boost the survival of *M. rubra* workers [61]. Conversely, sugars such as the monosaccharide xylose, which *M. rubra* strongly discriminated against (Fig. 6.5B), are not readily metabolized by ants [89,90] and reportedly increase mortality in cape bees, *Apis mellifera capensis* [91]. Our findings that both *C. modoc* and *M. rubra* discriminated against the disaccharides maltose and trehalose is somewhat surprising because *M. rubra* has the enzymatic ability of maltose breakdown [61], and trehalose generally helps regulate hemolymph sugar levels in insects [88]. However, trehalase – the enzyme capable of trehalose breakdown to its two glucose constituents – has been reported thus far only in the European thatching ant *Formica polytena* [92]. β-Galactosidase – the enzyme capable of melibiose break down – may occur in *M. rubra* [61], whereas α-

galactosidase and α-glucosidase – the enzymes capable of raffinose and melezitose breakdown, respectively [93] – have not yet been studied in aphid-tending ants although both enzymes occur in leafcutter ants [89,94]. Conceivably, the sugar solutions least consumed by *C. modoc* and *M. rubra* (Figs. 6.5–8) were discriminated against only in the presence of sought-after sugars such as fructose or sucrose, which were concurrently offered in multiple-choice experiments. Lacking a choice, ants might have consumed any sugar that they are capable of digesting. In turn, offering pest species of ants, such as *M. rubra*, a lethal bait containing their favorite sugar sucrose will likely improve bait uptake, transport to the nest, and trophallaxis with nestmates, thereby expediting the demise of nests.

Preferential consumption of certain sugar solutions (Figs.6.4–8) is the result of behavioural choices made by foraging ants that are dependent upon characteristics of the specific sugar solution (sugar type and concentration). In response to the sugar solution they encountered, individual ants decide how much to carry back in their crop to the nest [95–97], how many return trips to the resource to make [6], and how much (if any) trail pheromone to deposit [35,98–100]. More ants are recruited by strongly marked trails, ultimately leading to collective choices by ants for the most appealing resource [7].

Sugar concentration affecting consumption was clearly visible in our dose-response experiment (Fig. 6.4). Selecting fructose as a model sugar and testing solutions with increasing fructose concentration (5, 20, 40, 70%) for consumption by *C. modoc*, resulted in almost linearly increasing consumption rates (Fig. 6.4). However, while the ants preferentially consumed solutions with higher fructose content, the mechanisms underlying these feeding responses were not explicitly tested here. Solutions with higher fructose concentrations may have prompted foraging ants to take up larger crop loads, make more return trips to these resources, or to recruit more nest mates to them. The sugar concentration of resources does affect crop load of ants, but it is not necessarily the highest sugar concentration that elicits uptake of the largest crop load, as shown with the carpenter ant *Camponotus mus*, the Argentine ant, *Linepithema humile,* and the ponerine ant, *Odontomachus chelifer* [95–97]. Ants mark trails more intensely in response to more concentrated sugar solutions [98,99]. More *C. modoc* nest mates may have been recruited to high-dose fructose solutions, if foraging ants – on their return trip to the nest – deposited trail pheromone, and if recruited nest mates reinforced the trail with their own pheromone deposits. For example, having fed on more

concentrated sugar solutions, more worker ants of *Camponotus rufipes* pheromone-marked foraging trails [100]. Similarly, more than 90% of *L. niger* workers pheromone-marked trails after feeding on sucrose droplets that were greater than their crop volume [82], with fewer workers marking trails if they needed to feed on multiple sugar sources to fill their crop [101].

Sugar type, in addition to volume and concentration of sugar resources, also modulates the ants' trail marking propensity. For example, foragers of *L. niger* returning to the nest marked trails most intensely when they had fed on the aphid-derived sugars melezitose and raffinose, and on the common sugar sucrose [35]. Interestingly, motivation for simple sugars such as glucose and fructose may be motivated by their caloric content whereas melezitose may be due to their indication of a suitable aphid partner [102]. In our study, we kept the volume and concentration of sugar solutions constant to test for the effect of sugar type on consumption by *C. modoc* and *M. rubra*, revealing that sugar type and sugar consumption by ants are strongly linked (Figs. 6.5–8).

In conclusion, workers of *C. modoc* seek *C. splendens* honeydew for its sugar constituents rather than other macro- or micronutrients but their foraging decisions were not guided by aphid-specific sugars. Sucrose was a top-choice sugar for both *C. modoc* and *M. rubra* foragers likely due to its digestibility and nutritional value. While both sugar type and sugar concentration are ultimate causes for uptake of sugar sources by foraging ants, strong recruitment of nest mates to superior sugar sources is likely the major proximate cause.

6.6. Data availability

Data are available from the Dryad Digital Repository [103].

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6.9. Figures

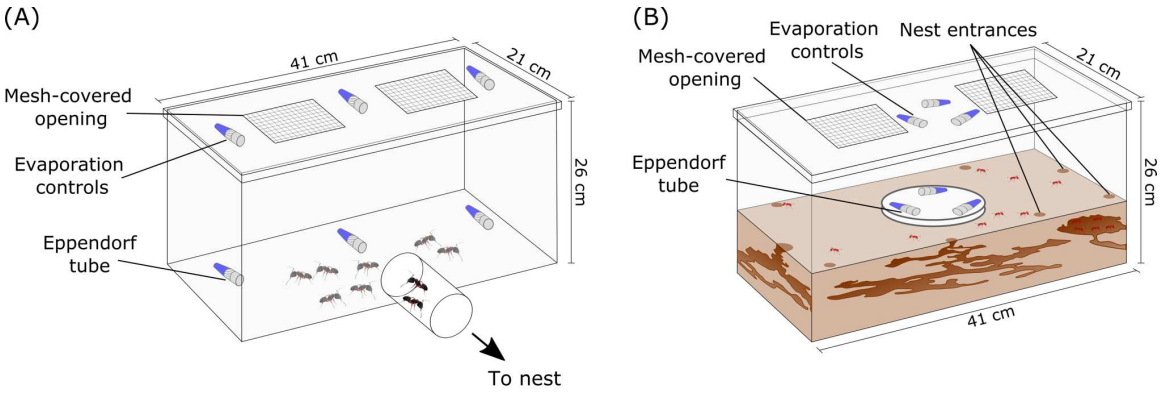

Figure 6.1. **Graphical illustrations of the experimental design used for testing foraging behavior of (A) Western carpenter ants, *Camponotus modoc*, and (B) European fire ants, *Myrmica rubra*, in response to concurrently offered aqueous sugar solutions [4.5-5% (w/v); shown in blue] retained with a cotton wick in Eppendorf tubes. The same stimuli were attached to arena lids inaccessible to ants to allow for measurements of passive water evaporation. Drawings not to scale.**

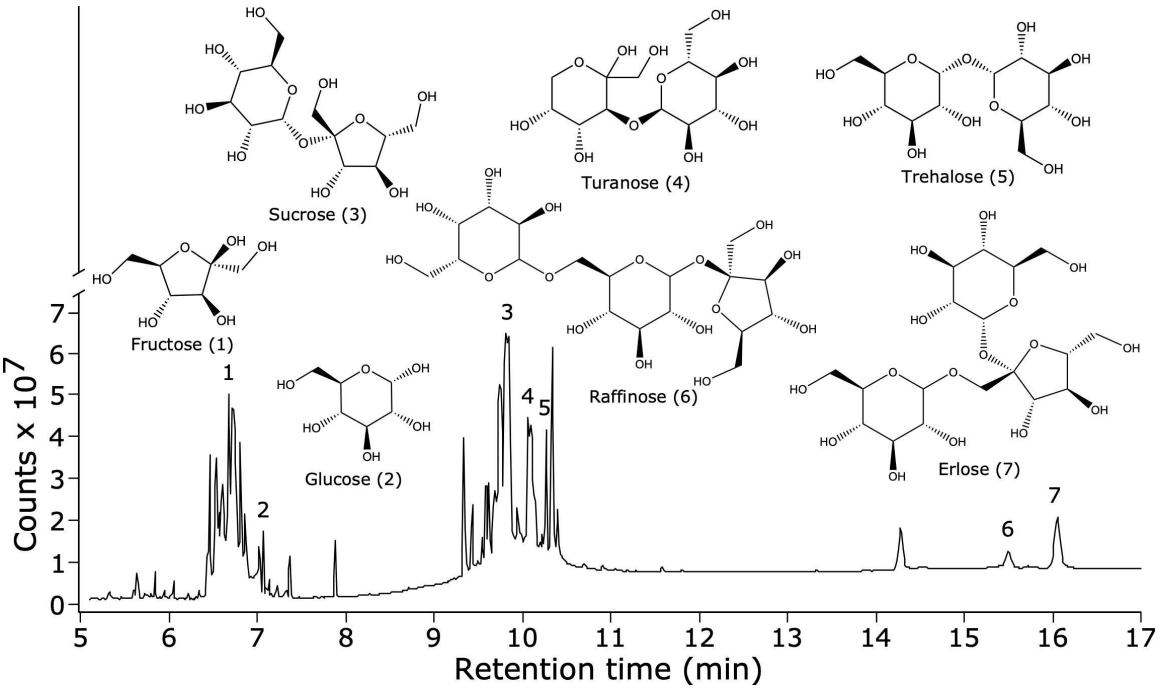

Figure 6.2. Total ion chromatogram of BSTFA-derivatized sugar constituents found in honeydew secretions of the aphid *Cinara splendens*. Note: derivatization of the polar sugar constituents with BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] allowed for gas chromatographic-mass spectrometric analyses of the BSTFA derivatives. Sugars are shown in underivatized form.

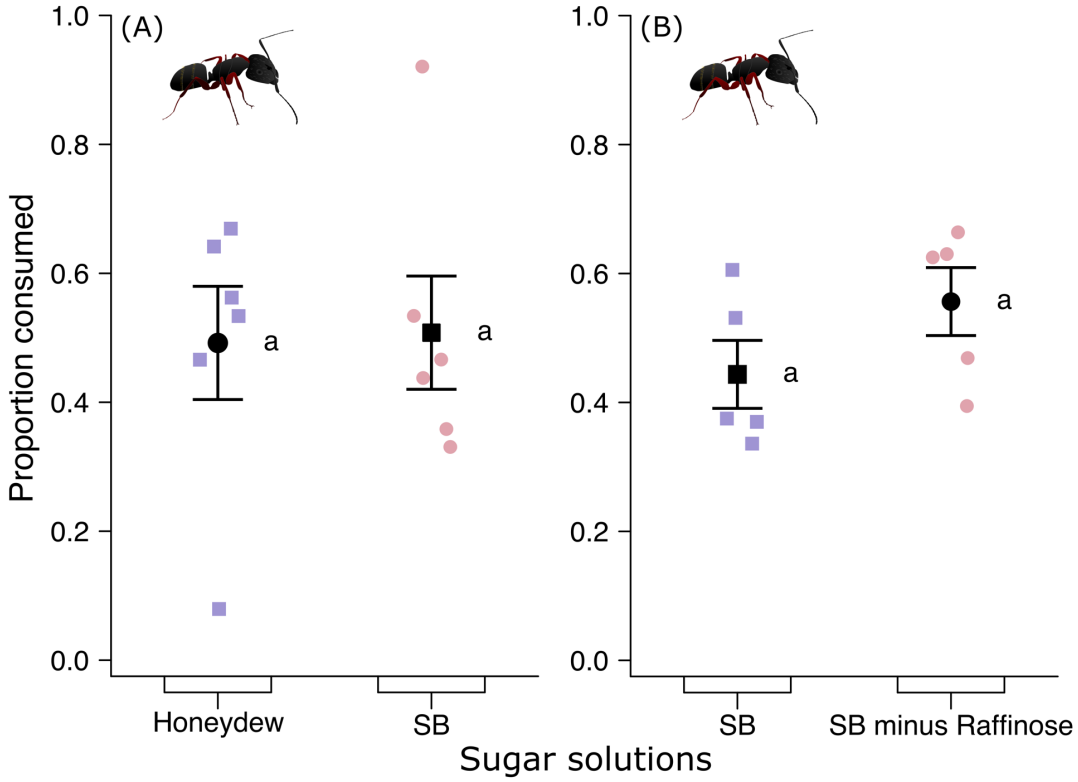

Figure 6.3. **Proportional consumption of test stimuli by foraging Western carpenter ants, *Camponotus modoc,* when offered a choice between aqueous solutions [4.5- 5% (w/v)] of (A) sugary honeydew secreted by *Cinara splendens* aphids and a synthetic blend (SB) containing sugar constituents at equivalent amount and ratio [fructose (14.3%), glucose (14.3%), sucrose (28.6%); trehalose (28.6%); raffinose (14.3%); see Fig. 3.2)], or (B) the same synthetic blend as in 'A' with or without the aphid-specific sugar raffinose. Stimuli were tested according to the experimental design shown in Figure 6.1. Coloured symbols show the data of individual replicates (6 nests in A; 5 nests in B) and black symbols the mean (± SE). For each panel, means labelled with the same letter are statistically not different from one another (likelihood ratio test: (A) $\chi^2 = 0.0196$, DF = 1, P = 0.889; (B) $\chi^2 = 2.521$, DF = 1, P = 0.112).

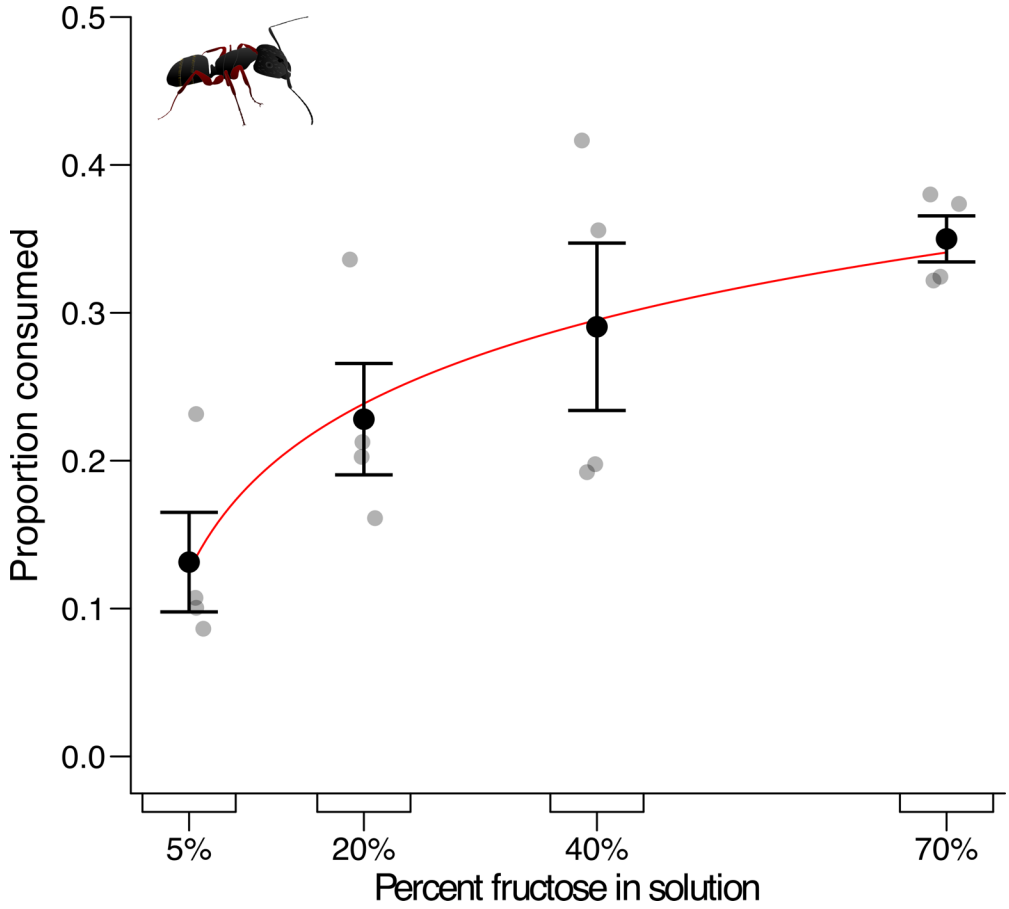

Figure 6.4. **Proportional consumption of test stimuli by foraging Western carpenter ants, *Camponotus modoc*. Test stimuli consisted of aqueous solutions containing fructose at different concentrations and were tested according to the experimental design illustrated in Figure 6.1. Grey symbols show the data of individual replicates (4 nests) and black symbols the mean (± SE). Higher fructose concentrations prompted larger consumption (likelihood ratio test, $\chi^2 = 14.152$, DF = 1, $P < 0.001$). Red line shows model-predicted values.**

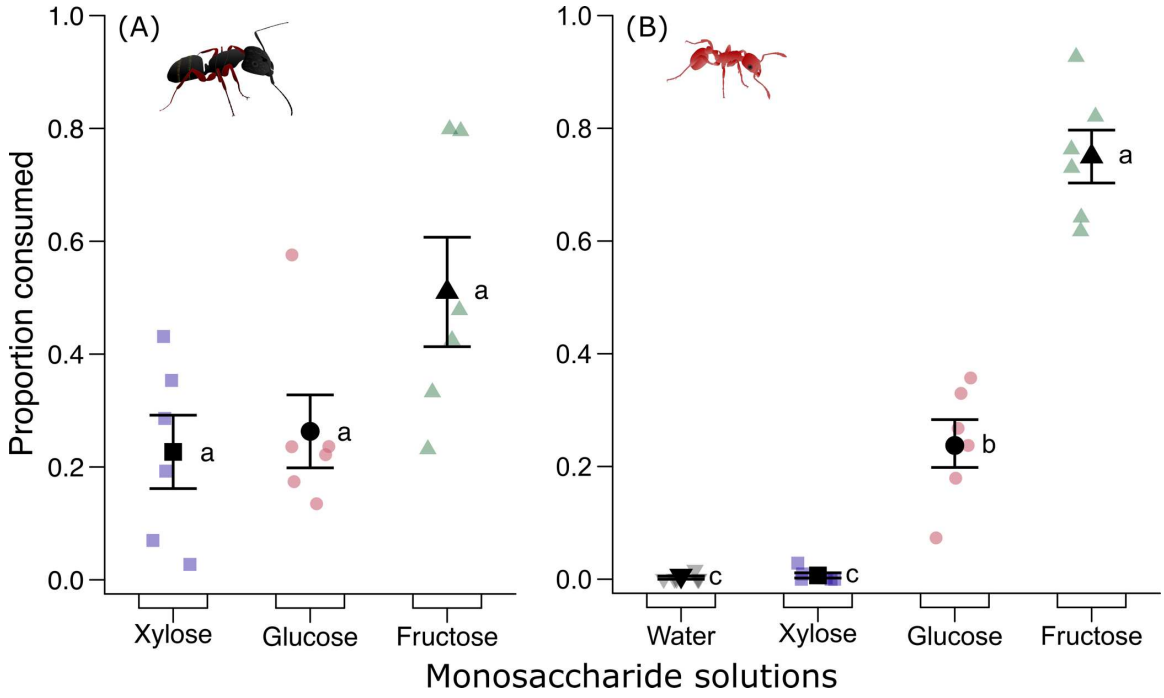

Figure 6.5. **Proportional consumption of test stimuli by foraging workers of (A) Western carpenter ants, *Camponotus modoc,* and (B) European fire ants, *Myrmica rubra*. Test stimuli consisted of aqueous solutions containing a monosaccharide at 5% (w/v) and were tested according to the experimental design illustrated in Figure 6.1. Coloured symbols show the data of individual replicates (6 nests each in A and in B) and black symbols the mean (± SE). Monosaccharide solutions prompted differential consumption by Western carpenter ants (likelihood ratio test: $\chi^2 = 7.7015$, DF = 2, P = 0.0213) and European fire ants (likelihood ratio test: $\chi^2 = 71.547$, DF = 3, P < 0.00001). For each panel, means labelled with different letters are statistically different from one another (Tukey HSD: P < 0.05).**

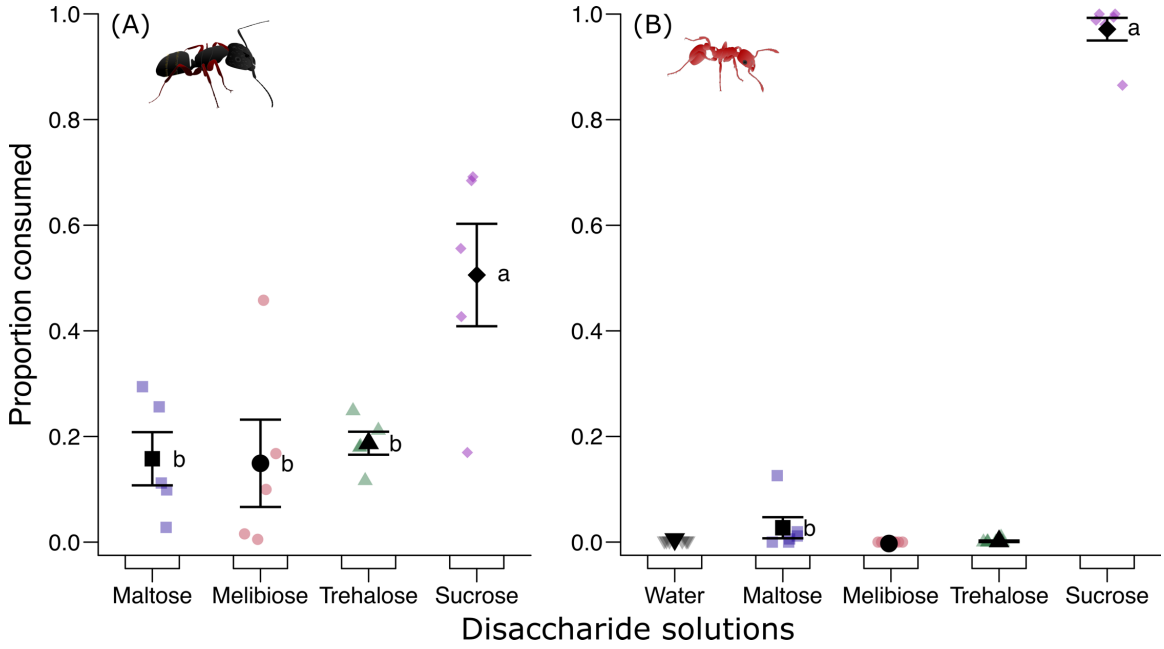

Figure 6.6. **Proportional consumption of test stimuli by foraging workers of (A) Western carpenter ants, *Camponotus modoc,* and (B) European Fire ants, *Myrmica rubra*. Test stimuli consisted of aqueous solutions containing a disaccharide at 5% (w/v) and were tested according to the experimental design illustrated in Figure 6.1. Coloured symbols show the data of individual replicates (5 nests in A; 6 nests in B) and black symbols the mean (± SE). Sugar solution treatments were excluded from statistical analyses if mean consumptions by ants were significantly less than zero (one sample T-test, $P < 0.05$; see Table C2). Disaccharide solutions prompted differential consumption by Western carpenter ants (likelihood ratio test: $\chi^2 = 15.239$, DF = 3, $P = 0.0016$) and European fire ants (likelihood ratio test: $\chi^2 = 55.82046$, DF = 1, $P < 0.00001$). For each panel, means labelled with different letters are statistically different from one another (Tukey HSD: $P < 0.05$).**

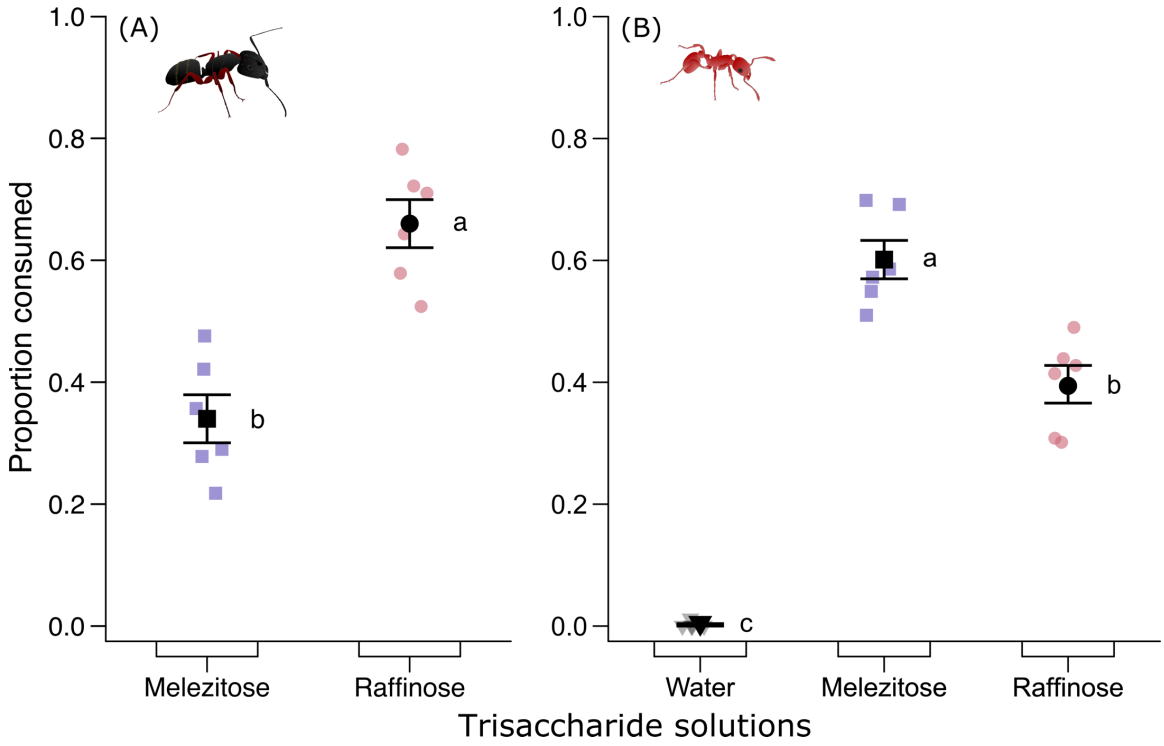

Figure 6.7. **Proportional consumption of test stimuli by foraging workers of (A) Western carpenter ants, *Camponotus modoc,* and (B) European fire ants, *Myrmica rubra.* Test stimuli consisted of aqueous solutions containing a trisaccharide at 5% (w/v) and were tested according to the experimental design illustrated in Figure 6.1. Coloured symbols show the data of individual replicates (6 nests each in A and in B) and black symbols the experimental mean (± SE). Trisaccharide solutions prompted differential consumption by Western carpenter ants (χ^2 = 17.498, DF = 1, P < 0.0001) and European fire ants (likelihood ratio test: χ^2 = 53.949, DF = 2, P < 0.00001). For panel B, means labelled with different letters are statistically different from one another (Tukey HSD: P < 0.05).**

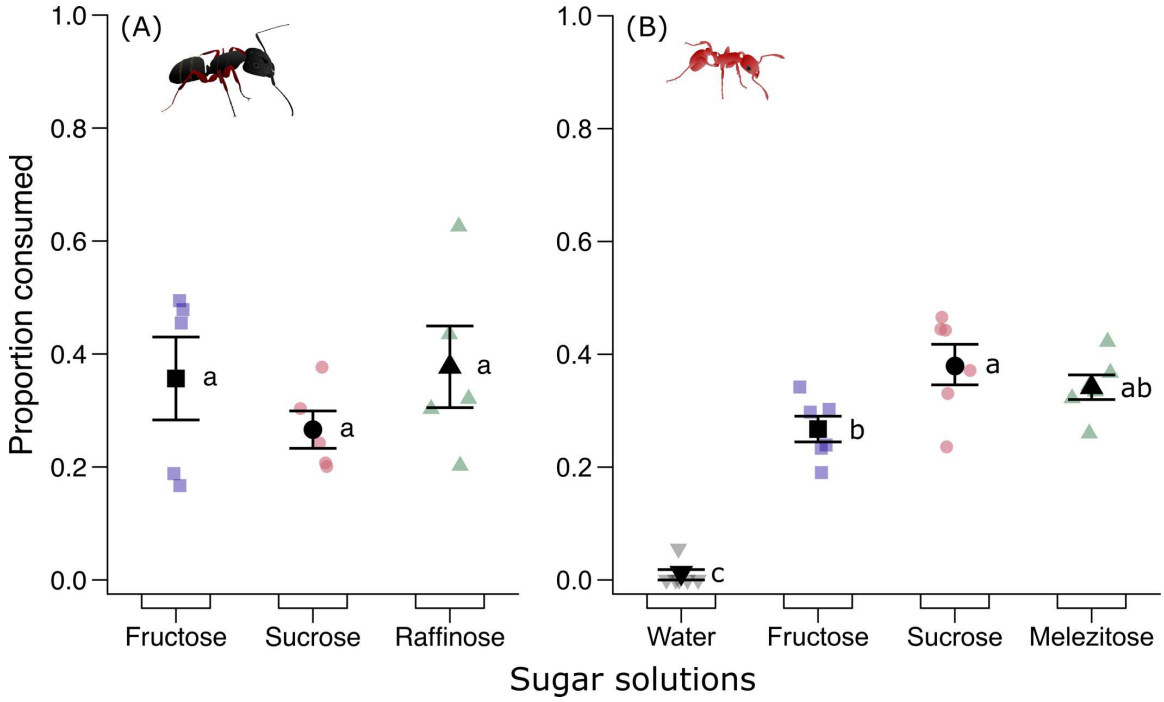

Figure 6.8. **Proportional consumption of test stimuli by foraging workers of (A) Western Carpenter ants, *Camponotus modoc,* and (B) European fire ants, *Myrmica rubra*. Test stimuli consisted of aqueous solutions containing the specific mono-, di-, or trisaccharide at 5% (w/v) favored by ants in preceding experiments (Figs. 5-7) and were tested according to the experimental design illustrated in Figure 6.1. Coloured symbols show the data of individual replicates (5 nests in A; 6 nests in B) and black symbols the experimental mean (± SE). Saccharide solutions prompted equal consumption by Western carpenter ants (likelihood ratio test: $\chi^2 = 2.0904$, DF = 2, P = 0.3516) but differential consumption by European fire ants (likelihood ratio test: $\chi^2 = 50.176$, DF = 3, P < 0.00001). For panel B, means labelled with a different letter are statistically different from one another (Tukey HSD: P < 0.05).**

Chapter 7.

Contrasting effects of amino acid types on foraging behaviour, colony growth, and worker mortality in European fire ants

A similar version of this chapter has been submitted: Renyard, A., Hoven, K., Gooding, G., Petrov, J., Chalissery, J.M., and Gries, G., (submitted). Contrasting effects of amino acid types on foraging behaviour, colony growth, and worker mortality in European fire ants. Myrmecological News.

7.1. Abstract

Foraging ants collect amino acids and proteins for developing larvae in their colony. Both essential amino acids (EAAs; some considered toxic to ants) and non-essential amino acids (non-EAAs) are important building blocks of proteins but EAAs cannot be synthesized by animals and must be obtained from their diet. Whether ants specifically forage for EAAs, and how EAAs affect ant colony growth, has rarely been investigated. Using European fire ants, *Myrmica rubra* and western carpenter ants, *Camponotus modoc,* as model species, we tested the hypotheses that (1) *M. rubra* and *C. modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs; (2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth; and (3) *M. rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (which are considered less toxic). In laboratory choice experiments, colonies of *M. rubra* and *C. modoc* preferentially foraged for EAAs rather than non-EAAs. Colonies of *M. rubra* that consumed both EAAs and non-EAAs, produced more larvae, but not more workers and queens, than colonies that consumed only EAAs or non-EAAs. In a mortality experiment with *M. rubra* workers that were removed from their colonies, workers that consumed sucrose and EAAs died sooner than workers that consumed sucrose and non-EAAs, possibly because they could not feed EAAs to larvae. Our results indicate that EAAs on their own, while critically important, are insufficient for ant colony growth. However, sucrose and EAAs as key macro-nutrients should be offered in highly appealing baits for control of pest ants.

7.2. Introduction

Cooperative brood care is one of the defining features of eusocial insect societies including ants (WILSON 1971). Worker ants, the non-reproductive cast of ant colonies, engage in nest construction, colony defense, grooming, and foraging to provide care for the colony's developing larvae and pupae (HÖLLDOBLER & WILSON 1990). The brood care by workers contributes to colony growth and produces reproductive queen ants that disperse and start new colonies (HÖLLDOBLER & WILSON 1990). While workers themselves do not reproduce, they provide allo-parental care, ultimately increasing their own fitness by the passing of shared genes through their queen (KORB & HEINZE 2008).

Worker ants sense, and behaviourally respond to, the needs of the colony's brood. They monitor the brood's well-being, keeping brood in suitable microclimates, removing pathogens, and providing adequate nutrition. Worker ants sense the brood's abiotic conditions, such as temperature (PORTER & TSCHINKEL 1993, ROCES & NUNEZ 1995, ANDERSON & MUNGER 2003, PENICK & TSCHINKEL 2008, KARLIK & al. 2016), humidity (Potts & al., 1984 but see Karlik & al., 2016), and CO_2 (RÖMER & al. 2018), and move brood to optimal growing conditions. Microbial pathogens on brood (KARLIK & al. 2016) prompt workers to engage in mechanical grooming and chemical secretions to remove or kill these microbes (UGELVIG & al. 2010, TRAGUST, MITTEREGGER, & al. 2013, TRAGUST, UGELVIG, & al. 2013). Lastly, the presence of brood shifts the behaviour of foragers, mobilizing them (PORTHA & al. 2002) to collect more food and more protein (CORNELIUS & GRACE 1997, DUSSUTOUR & SIMPSON 2008, DUSSUTOUR & SIMPSON 2009).

Workers collect the nutrients that the brood requires. Ant larvae signal hunger to ant nurses (CREEMERS & al. 2003, KAPTEIN & al. 2005, PEIGNIER & al. 2019) that, in turn, then solicit food from foragers (SORENSEN & VINSON 1981, SORENSEN & al. 1985). While worker ants require primarily carbohydrates (GROVER & al. 2007, COOK & al. 2010, DUSSUTOUR & SIMPSON 2012, SHIK & SILVERMAN 2013, BAZAZI & al. 2016, ARGANDA & al. 2017, WITTMAN & al. 2018), developing larvae require additionally more proteins (PORTER 1989, EVANS & PIERCE 1995, FELDHAAR & al. 2007, GROVER & al. 2007, SHIK & SILVERMAN 2013). Whole proteins are obtained from prey and deceased insects, whereas free amino acids are obtained from sources such as plant nectar (BLÜTHGEN & al. 2004, GONZÁLEZ-TEUBER & HEIL 2009, SHENOY & al. 2012), aphid honeydew

(BLÜTHGEN & al. 2004, WOODRING & al. 2004, SHAABAN & al. 2020), and insect hemolymph (KANOST 2009). Developing ant larvae metabolize proteins to amino acids, the building blocks of new proteins (CHAPMAN 2013, COHEN 2015). Generally, organisms incorporate 20 proteogenic (protein creating) amino acids in protein biosyntheses, and use non-proteogenic amino acids (e.g., γ-aminobutyric acid) for other functions such as signalling (CHAPMAN 2013, COHEN 2015). Some animals can synthesize non-essential amino acids (non-EAAs) but must obtain essential amino acids (EAAs) from their diet (CHAPMAN 2013, COHEN 2015). In insects, 8–10 amino acids are typically deemed essential (CHAPMAN 2013, COHEN 2015). Some ant taxa obtain both EAAs and non-EAAs through their gut microbiota that are capable of converting nitrogenous waste products to amino acids. For example, carpenter ants, *Camponotus* spp., and turtle ants, *Cephalotes* spp., harbour gut microbes that convert urea, or uric acid, to amino acids (FELDHAAR & al. 2007, HU & al. 2018). However, the effects of EAAs and non-EAAs on ant colony growth have yet to be rigorously investigated. Colonies of *Camponotus floridanus* that were provisioned with a diet lacking EAAs and containing antibiotics (to kill amino acid-synthesizing microbes) raised fewer pupae (FELDHAAR & al. 2007).

As most ants obtain EAAs from their diet, it follows that they are able to distinguish between EAAs and non-EAAs. Generally, many ants prefer nutrient solutions containing amino acids (LANZA & KRAUSS 1984, BLÜTHGEN & FIEDLER 2004, GONZÁLEZ-TEUBER & HEIL 2009), particularly at higher concentration (LANZA 1991, LANZA & al. 1993, GONZÁLEZ-TEUBER & HEIL 2009), but there is no consistent preference for specific amino acids. Some but not all ants preferentially feed on EAAs (SHENOY & al. 2012). Argentine ants, *Linepithema humile*, prefer EAAs to non-EAAs for consumption, and when deprived of a single EAA preferentially forage for that EAA (Csata & al., 2020). Interestingly, *L. humile* workers consuming the EAAs methionine, threonine and phenylalanine had a shorter lifespan than workers consuming other proteogenic amino acids (ARGANDA & al. 2017), suggesting that these EAAs are toxic to ants.

Here we worked with western carpenter ants, *Camponotus modoc,* and European fire ants, *Myrmica rubra,* as model species. *Camponotus modoc* is native to the west coast of North America, excavating nests in the wood of conifers (HANSEN & KLOTZ 2005). *Myrmica rubra* is an aggressive, stinging, and soil-nesting ant (WETTERER & RADCHENKO 2010) that is native to Eurasia but has invaded coastal areas of eastern and western North America (WETTERER & RADCHENKO 2010). We tested three

hypotheses: (1) *M. rubra* and *C. modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs; (2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth; and (3) *M. rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs.

7.3. Materials and methods

7.3.1. Maintenance of ant colonies:

Colonies of *C. modoc* were collected and maintained as previously reported (RENYARD & al. 2019). Briefly, nests were excised from conifer logs and stumps, and placed in large plastic bins (64 × 79 × 117 cm) that were kept in an outdoor undercover area, where they experienced natural temperature and light cycles. Bins were connected to glass containers via barbed plumbing fixtures and Nalgene™ tubing. Ants were provisioned with 20% (w/v) sugar water, cockroaches, meal worms, and apples *ad libitum*.

Colonies of *M. rubra* were collected and maintained similar to previous reports (HOEFELE & al. 2021, RENYARD & al. 2021). Six *M. rubra* nests were excavated at Inter River Park (North Vancouver, BC, Canada) and placed, together with nesting soil, in glass containers (26 × 21 × 40.6 cm) or plastic bins (41 × 29 × 24 cm) kept indoors at 25 °C and at a 16:8 light dark cycle. Nests were sprayed with water several times per week, and ants were fed the same diet as *C. modoc* (see above).

7.3.2. Preparation of liquid nutrient solutions - general descriptions:

Liquid nutrient solutions (see Table 7.2 for nutrient compositions) were prepared by weighing dry ingredients (TR 204 scale; Denver Instrument Company, CO, USA), and then mixing them in distilled water. Aliquots (1 mL) of these solutions were pipetted into 1.5-mL Eppendorf tubes (Thermo Fisher Scientific, Waltham, MA 02451, USA) which were stored in a freezer (-4 °C) until needed.

For testing H1, that colonies with brood preferentially forage for EAAs rather than non-EAAs, amino acids were deemed either EAAs or non-EAAs, as listed in Feldhaar &

al. (2007) and based on personal communication with Feldhaar. For testing H2, that colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth, the diet was prepared as described in Straka & Feldhaar (2007), but some unavailable ingredients were omitted (see Table 7.2). For testing H3, that worker ants feeding on EAAs die sooner than worker ants feeding on non-EAAs, EAAs or non-EAAs were combined with sucrose, a preferred sugar of *C. modoc* and *M. rubra* (RENYARD & al. 2021).

Prior to experiments, Eppendorf tubes were removed from the freezer to thaw, and then vortexed to dissolve all solutes. Then, a 1-cm long piece of cotton dental wick (Richmond Dental & Medical, Charlotte, NC 28205, USA) was inserted into each tube, thus allowing ants to ingest the liquid without spillage.

7.3.3. Specific experiments

(H1) M. rubra and C. modoc colonies with brood preferentially forage for EAAs rather than non-EAAs (Exps. 1–4):

All experiments followed an established protocol (RENYARD & al. 2021) with slight modifications. Prior to bioassays, we deprived *C. modoc* colonies of cockroaches and apples, and of sugar water for 24 h and 4 h, respectively. Bioassays were run in plexiglass containers (50.5 × 30.5 × 33 cm; Fig. 7.1a) covered by a lid with mesh holes to allow ventilation. To prevent ant escape, the upper inner container walls were coated with an equal mix of Vaseline (Unilever, London, UK) and paraffin oil (Anachemia, Lachine, QC H8R1A3, CA). For each experiment, we prepared a set of tubes for nutrient consumption by ants and another set of tubes for monitoring passive water evaporation ("evaporation controls"). All Eppendorf tubes were weighed prior to bioassays. Eppendorf tubes were taped, with positions randomly assigned and spaced equidistantly in an arc, to the arena bottom, 22 cm away from the container entrance hole. Corresponding evaporation control tubes were taped to a plexiglass platform suspended from the container lid. To initiate a replicate, tubes were uncapped and each container was connected via Tygon® tubing (diam.: 2.54 cm) and barbed plumbing connectors (diam.: 2.54 cm) to a *C. modoc* housing bin, allowing ants to freely forage in the container. Bioassay replicates were run for 4 h but were terminated sooner if ants had completely consumed the test solution of any one tube. At the end of each replicate, tubes were reweighed to determine consumption by ants and the amount of water

evaporation. Bioassay containers were cleaned with hexane and ethanol (70%), and plumbing fixtures and Tygon® tubing were washed with soapy water.

All bioassays with *M. rubra* colonies (food-deprived 24 h) were run in their nesting containers (Fig. 7.1b). Prior to each bioassay replicate, all Eppendorf tubes were weighed, and tubes with nutrients for consumption by ants were randomly assigned to the edge of a jar lid (diam: 15 cm), whereas corresponding evaporation control tubes were taped, inaccessible to ants, to the underside of container lids. Bioassay replicates were initiated by uncapping all Eppendorf tubes, and placing the jar lids with Eppendorf tubes on the soil surface inside bioassay containers. Bioassays were run for 6 h but were terminated sooner if ants had consumed the entire nutrient solution in an Eppendorf tube. Tubes were capped and reweighed after replicates to determine nutrient consumption by ants. Jar lids were cleaned with soapy water between experiments.

To test H1, colonies in experiments 1 (*C. modoc*) and 2 (*M. rubra*) were offered three choices: (1) 11 EAAs + 10 non-EAAs (1.05% total w/v) in water; (2) 10 non-EAAs (0.5%) in water; and (3) a water control. Additionally, colonies in experiments 3 (*C. modoc*) and 4 (*M. rubra*) were offered: (1) 11 EAAs + 10 non-EAAs (1.05%) in water; (2) 11 EAAs (0.55%) in water; and (3) a water control.

(H2) M. rubra colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth (Exp. 5):

To test H2, *M. rubra* colonies were collected at Inter River Park, and sorted into 30 small lab colonies, each consisting of 30 workers and two queens housed in small plastic containers (17 × 17 × 6 cm) with a mesh-covered hole in the lid for air exchange (Fig. 7.1c). Each container was fitted with a 10-mL test tube (13 × 100 mm) filled halfway with water and plugged with a cotton ball to provide a humid environment. Colonies were provisioned with one of three types of nutrient blends. All blends contained sucrose, salts, and vitamins (Table 7.2) but differed in amino acid composition: (1) 11 EAAs + 10 non-EAAs (2.1%); (2) 10 non-EAAs (1.0%); and (3) 11 EAAs (1.1%). All blends were provided in Eppendorf tubes and replaced twice per week. After 16 weeks, each colony was frozen, and larvae, worker ants, queen ants were counted.

(H3) M. rubra workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (Exp. 6):

To test H3, worker mortality was tracked over time in response to consumption of specific macro-nutrients: (1) aqueous sucrose alone, (2) aqueous sucrose plus 11 EAAs, and (3) aqueous sucrose plus 10 non-EAAs. Colonies were field collected, sorted and housed as described (see H2) but each colony contained only 30 workers without queens. Each nutrient solution was provided in Eppendorf tubes and replaced twice per week. Every week, dead workers in each colony were counted.

7.3.4. Statistical analysis:

We analysed data for experiments using generalized linear models (GLM) and generalized linear mixed models (GLMM). For experiments 1–4, we calculated consumption of nutrient solutions by ants by subtracting the weight loss in evaporation controls from the weight loss of nutrient test solutions. In some replicates with little ant colony activity, nutrient test solutions had a slightly negative value following weight loss subtraction due to water evaporation. As there could not be 'negative feeding' on a nutrient solution by a colony, we considered these values to be a zero. We filtered non-responding colonies by including only nests which had at least one positive consumption value. To account for differences in ant colony activity, we analysed proportions rather than absolute amounts. To calculate proportional consumption, we divided the consumption value of a given treatment (nutrient solution) by the total amount of consumption by a colony. As we used a GLMM with a beta distribution, we applied a standard transformation to restrict our data between the interval 0 and 1 (SMITHSON & VERKUILEN 2006). We fit proportional consumption as our response variable and treatment as the predictor variable, with ant colony as a random intercept. In experiment 5, we used a GLM to model the number of larvae with a negative binomial distribution, and the number of queens and workers using a Conway-Maxwell Poisson distribution with treatment as our predictor variable. For experiment 6, we fit the proportion of live ants as a binomial GLMM with a treatment by day interaction, and colony as a random intercept. For all models, we assessed the significance of each predictor using a likelihood ratio test (LRT), and ran Tukey adjusted pairwise comparisons between means (Exps. 1–5).

We used R (v. 4.2.2) and R studio (v. 2022.07.1+554) to analyze data and produce graphics (R CORE TEAM 2022). We processed data using the tidyverse packages (WICKHAM & al. 2019) and the plyr package (WICKHAM 2011). We used the glmmTMB package (BROOKS & al. 2019) to fit models, and the DHARMa package to inspect their fit (HARTIG 2022). We obtained estimated marginal means and 95% confidence intervals using the emmeans package (LENTH 2023). We graphed data using the ggplot2 package (WICKHAM 2016) and Inkscape for final figure assembly(v. 1.0.2). Data and code can be accessed at RENYARD & al. (2023).

7.4. Results

7.4.1. (H1) *M. rubra* and *C. modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs (Exps. 1–4):

In experiments 1 and 2, the composition of amino acid blends (11 EAAs + 10 non-EAAs or 10 non-EAAs only) significantly affected consumption by *C. modoc* (LRT: $\chi^2 = 21.59$, d. f. = 2, $p < 0.0001$) and *M. rubra* (LRT: $\chi^2 = 28.279$, d. f. = 2, $p < 0.0001$). Ants consumed blends containing both EAAs and non-EAAs significantly more than blends containing only non-EAAs, which were ingested as little as the water control (Tukey adjusted p-value < 0.05; Table D1; Fig. 7.2).

In experiments 3 and 4, the composition of amino acid blends (11 EAAs + 10 non-EAAs or 11 EAAs only) again significantly affected consumption by *C. modoc* (LRT: $\chi^2 = 23.68$, d. f. = 2, $p < 0.0001$) but not by *M. rubra* (LRT: $\chi^2 = 5.6$, d. f. = 2, $p = 0.06$). Colonies of *C. modoc* consumed the blend of EAAs and non-EAAs only slightly more (but statistically significant) than the blend of EAAs, both blends being consumed more than water (Tukey adjusted p-value < 0.05; Table D1; Fig. 7.2). In contrast, colonies of *M. rubra* consumed the blend of EAAs and non-EAAs as much as the blend of EAAs, and they ingested both blends numerically (but not statistically) more than the water control (Tukey adjusted p-value > 0.05; Table D1; Fig. 7.2).

7.4.2. (H2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth (Exp. 5):

The composition of the amino acid blend [(1) 11 EAAs + 10 non-EAAs; (2) 11 EAAs; or (3) 10 non-EAAs] significantly affected the number of ant larvae (LRT: χ^2 = 21.48, d. f. = 2, p <0.0001) but not the number of worker ants (LRT: χ^2 = 5.04, d. f. = 2, p = 0.08) and queen ants (LRT: $\chi^2 = 0.36$, d. f. = 2, p = 0.83) present in colonies. Colonies provisioned with both EAAs and non-EAAs produced more larvae than colonies provisioned with either EAAs or non-EAAs, with the latter two colonies having similarly few larval offspring (Tukey adjusted p value < 0.05; Table D2; Fig. 7.3).

7.4.3. (H3) *M. rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (Exp. 6):

Nutrient blend [(1) sucrose + 11 EAAs; (2) sucrose + 10 non-EAAs; (3) sucrose only] was a significant predictor of worker mortality, day in experiment, and interaction between mortality and day (mortality: $\chi^2 = 19.13$, d. f = 4, p = 0.0007; day: $\chi^2 = 2269.4$, d. f. = 3, p < 0.0001; interaction: $\chi^2 = 9.29$, d. f. = 2, p = 0.01; Fig. 7.4). The negative interaction term between 'day' and 'EAAs', and the positive interaction term between 'non-EAAs' and 'day', indicate that over time worker ants consuming EAAs died the fastest, followed by workers consuming sucrose, and sucrose plus non-EAAs (RENYARD & al. 2023)

7.5. Discussion

Our data support the hypotheses that *M. rubra* and *C. modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs, and that *M. rubra* workers feeding on EAAs die sooner than workers feeding on non-EAAs. However, our data do not support the hypothesis that *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth. Below we elaborate on these results.

There is increasing evidence that some ant taxa preferentially forage for EAAs rather than non-EAAs (Shenoy & al., 2012; Csata & al., 2020; this study). In our study,

the presence of EAAs stimulated feeding by both *C. modoc* and *M. rubra,* whereas non-EAAs were as unappealing as water. For *C. modoc,* but not *M. rubra,* a blend of both EAAs and non-EAAs was more appealing than the EAA-only blend, revealing a contributing effect of non-EAAs to the overall blend 'appeal'. That the equivalent effect was not observed with *M. rubra* colonies may be due, in part, to their highly variable feeding responses. Preferential foraging for EAAs was also reported in studies with *L. humile* (CSATA & al. 2020) and with white-footed ants, *Technomyrmex albipe*s, but not with bicoloured arched ants, *Myrmicaria brunnea* (SHENOY & al. 2012). Our findings and those reported for *L. humile* indicate that ants retain their EAA preference even when, experimentally, they have been deprived of all amino acids and proteins. This selective consumption of EAAs is perplexing considering that a lack of either EAAs or non-EAAs resulted in smaller broods (Fig. 7.3). An explanation may lie in the scarceness of EAAs in the ants' food sources and the difficulty to obtain them. In contrast to non-EAAs, EAAs are deemed not to be abundantly and reliably present in ant food sources such as extra-floral and floral nectar and aphid honeydew (Blüthgen & al., 2004; Woodring & al., 2004; Shenoy & al., 2012; Shaaban & al., 2020 but see González-Teuber & Heil, 2009). Although proteins of insect prey generally contain both EAAs and non-EAAs (CHAPMAN 2013), and insect hemolymph contains all 20 amino acids (KANOST 2009), *C. modoc* foragers rarely return to the nest with insect prey (TILLES & WOOD 1986), which would result in limited supply of EAAs for nestmates and brood. Selective consumption of EAAs by *C. modoc* and *M. rubra* may ultimately be motivated by the scarceness of these amino acids in the regular food sources of these ants.

Contrary to our prediction, both EAAs and non-EAAs contributed to the brood size of colonies (Fig. 7.3). Even though insects can synthesize non-EAAs, a large deficiency of non-EAAs may impair development and growth (CHAPMAN 2013). Lack of even a single EAA in an insect's diet can greatly reduce developmental growth (HOUSE 1961, CHAPMAN 2013). Although necessary for insect growth, EAAs on their own are commonly insufficient, and specific non-EAAs are needed to improve growth. In honey bees, *Apis mellifera,* EAAs as dietary constituents improve brood development but not overall colony growth (HENDRIKSMA & al. 2019). Larvae of *Culex pipiens* mosquitoes developed poorly on a diet containing just the 12 EAAs, but diets containing all amino acids, or just the non-EAAs glycine or serine, equally improved larval development (DADD 1978). Similarly, caterpillars of the silkworm, *Bombyx mori,* developed better on a

diet also containing non-EAAs (ITO & ARAI 1967). That the gut endosymbionts of *Cephalotes* and *Camponotus* ants provide their ant hosts with both EAAs and non-EAAs (FELDHAAR & al. 2007, HU & al. 2018) exemplifies the physiological importance of either amino acid type.

Proteins and amino acids are vital for ant colony growth (PORTER 1989, EVANS & PIERCE 1995, FELDHAAR & al. 2007) but are toxic to worker ants (Cook & al., 2010; Dussutour and Simpson, 2012; Bazazi & al., 2016; Arganda & al., 2017; this study). Particularly harmful to worker ants are the EAAs methionine, threonine and phenylalanine, and the non-EAA serine (ARGANDA & al. 2017). In our study (Fig. 7.4), ants consuming sucrose and EAAs died sooner than ants consuming sucrose and non-EAAs, with the latter group of ants living longer than ants consuming only sucrose. These data imply that non-EAAs help maintain vital physiological functions in ants. The toxic effects of amino acids or proteins on workers, as shown in our study and others (COOK & al. 2010, DUSSUTOUR & SIMPSON 2012, ARGANDA & al. 2017), may be attributable to the experimental design which restricted the ants' diet to these macro-nutrients over a relatively long time scale (~5-100 days). This explanation seems plausible because the deleterious effects of amino acids or proteins on worker ants were ameliorated or not observed when workers could pass these amino acids to brood via trophallaxis (DUSSUTOUR & SIMPSON 2009, ARGANDA & al. 2017), and when colonies were offered choices between nutritionally diverse food sources (BAZAZI & al. 2016). Our interpretation that brood provisioning motivates preferential EAA consumption by worker ants is supported by distinct morphological and physiological characteristics of ants. Their narrow petiole physically restricts protein movement through the digestive tract (HÖLLDOBLER & WILSON 1990) and their low protease activity (PETRALIA & al. 1980, ERTHAL & al. 2007) slows protein breakdown.

With the physical and metabolic inability of worker ants to process proteins and amino acids, their quest for EAAs is clearly motivated by brood provisioning. Although both *C. modoc* and *M. rubra* colonies preferentially foraged for EAAs, non-EAAs were still needed to increase brood size in *M. rubra* colonies. It follows that preferential foraging for EAAs is likely driven by their relative scarceness in the ants' food sources and the ants' inability to synthesize EAAs. Our data also indicate that nutrient consumptions by ants in feeding trials do not necessarily reveal the complement of macro- and micro-nutrients that thriving ant colonies require, and that the effect of

dietary constituents on ant colony functioning and growth must be investigated in proper context and long-term studies.

Lastly, our findings have significant implications for control of (invasive) pest ants. We have previously shown that ant baits both containing apples (carbohydrates) and mealworms (proteins, amino acids) elicit stronger foraging responses by *M. rubra* colonies than either apples or mealworms alone (HOEFELE & al. 2021). We have also argued that key carbohydrates and amino acids should be identified so that they can be incorporated in ant baits with extended shelf life (HOEFELE & al. 2021). We have made progress toward this goal when we found that sucrose is a preferred sugar for both *C. modoc* and *M. rubra* (RENYARD & al. 2021), as also reported for other ants (CORNELIUS & al. 1996, BLÜTHGEN & FIEDLER 2004, ZHOU & al. 2015, RENYARD & al. 2021). Here, we show that both *C. modoc* and *M. rubra* preferentially consumed EAAs, and that non-EAAs – added to EAAs – only marginally improved the bait's appeal, suggesting that non-EAAs can be omitted as bait constituents. Combining sucrose (the preferred carbohydrate) with EAAs (the preferred amino acids) in the same bait would make it appealing to both sugar- and protein-loving ants, and would retain its season-long appeal to foraging pest ants, even if they were to shift their macro-nutrient preference (e.g., Abbott & al., 2014) over the foraging season from carbohydrates to proteins, or *vice versa.*

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7.8. Tables

Table 7.1. Hypotheses (H) tested, and experiments and replicates (n) run, with colonies of *Camponotus modoc* and *Myrmica rubra*.

Exp. #	Stimuli[a] tested	Species tested (n)
(H1) Colonies with brood preferentially forage for EAAs[c] rather than non-EAAs[d]		
1–2	EAAs + non-EAAs (1.05%[b]) vs non-EAAs (0.5%) vs Water	C. modoc (6); M. rubra (5)
3–4	EAAs + non-EAAs (1.05%) vs EAAs (0.55%) vs Water	C. modoc (5); M. rubra (6)
(H2) Colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth		
5	EAAs + non-EAAs (2.1%) vs EAAs (1.1%) vs non-EAAs (1.0%)	M. rubra (10)
(H3) Workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs		
6	Sucrose (5.55%) vs Sucrose (4.55%) + EAAs (1%) vs Sucrose (4.55%) + non-EAAs (1%)	M. rubra (10)

[a]For blend compositions and chemical suppliers of nutrients see Table 7.2

[b]Percentages are expressed as weight by volume (w/v)

[c]EAAs = essential amino acids

[d]non-EAAs = non-essential amino acids

Table 7.2. List of macro-nutrients [sucrose, essential amino acids (EAAs), non-essential amino acids (non-EAAs)], micro-nutrients (salts, vitamins, others), and their chemical purities, suppliers, and chemical abstract service (CAS) numbers, used in test stimuli.

Nutrients	Chemicals	Fraction of total	Supplier[a]	% Purity	CAS
Sucrose	D-sucrose	1.00	SA	≥99	57-50-1
non-EAAs[b]	L-asparagine	0.1	SA	≥99	5794-13-8
	L-aspartic acid	0.1	SA	≥98	56-84-8
	L-cysteine	0.1	SA	≥97	52-90-4
	L-glutamine	0.1	SA	≥99	56-85-9
	L-glycine	0.1	SA	≥98	56-40-6
	L-lysine	0.1	SA	≥98.5	56-87-1
	L-proline	0.1	SA	≥99	147-85-3
	L-serine	0.1	SA	≥99	56-45-1
	L-tyrosine	0.1	AC	≥98	60-18-4
	γ-amino butyric acid	0.1	SA	≥99	56-12-2
EAAs	L-glutamic acid	0.091	SA	99	56-86-0
	L-alanine	0.091	SA	≥98	56-41-7
	L-isoleucine	0.091	MI	≥98	73-32-5
	L-leucine	0.091	SA	97	61-90-5
	L-valine	0.091	SA	≥98	72-18-4
	L-tryptophan	0.091	SA	≥98	73-22-3
	L-arginine	0.091	SA	≥98	74-79-3
	L-histidine	0.091	SA	≥99	71-00-1
	L-threonine	0.091	SA	≥98	72-19-5
	L-methionine	0.091	SA	≥98	63-68-3
	L-phenylalanine	0.091	SA	99	63-91-2
Salts	$CuCl_2$	0.0002	SA	97	7447-39-4
	$FeCl_3$	0.0019	OW	98	7705-08-0
	$MnCl_2$	0.0004	SA	≥99	7773-01-5
	NaCl	0.0020	FI	≥99	7647-14-5
	$ZnCl_2$	0.0008	AA	≥99	7646-85-7
	KH_2PO_4	0.5054	SA	≥99	7778-77-0
	$MgSO_4$	0.4892	CL	≥99	7487-88-9
Vitamins/MISC	*p*-amino benzoic acid	0.0424	SA	≥99	150-13-0
	ascorbic acid	0.4235	BS	≥97	50-81-7
	biotin	0.0004	SA	≥99	58-85-5
	calcium D-pantothenate	0.0212	SA	≥98	137-08-6
	folic acid	0.0042	SA	≥97	59-30-3
	nicotinic acid	0.0424	SA	≥98	59-67-6

pyridoxin hydrochloride	0.0106	SA	≥	98	58-56-0
riboflavin	0.0212	SA	≥	98	83-88-5
thiamine	0.0106	SA	≥	99	67-03-8
meso-inositol	0.2118	SA	≥	99	87-89-8
choline chloride	0.2118	SA	≥	99	67-48-1

[a] SA = Sigma Aldrich, Burlington, MA, USA; AC = Anachemia Canada Inc., Lachine, Quebec, Canada; MI = Millipore, Burlington, MA, USA; OW = Oakwood Products, Inc., Estill, South Carolina, USA; FI = Fisher Scientific International, Inc., Pittsburgh, PA, USA; AA = Alfa Aesar, Ward Hill, MA, USA; CL Caledon Laboratories Ltd., Georgetown, ON, Canada; BS = Bio-Serv, Flemington, NJ, USA.

[b] Amino acids listed as in Feldhaar & al. (2007).

7.9. Figures

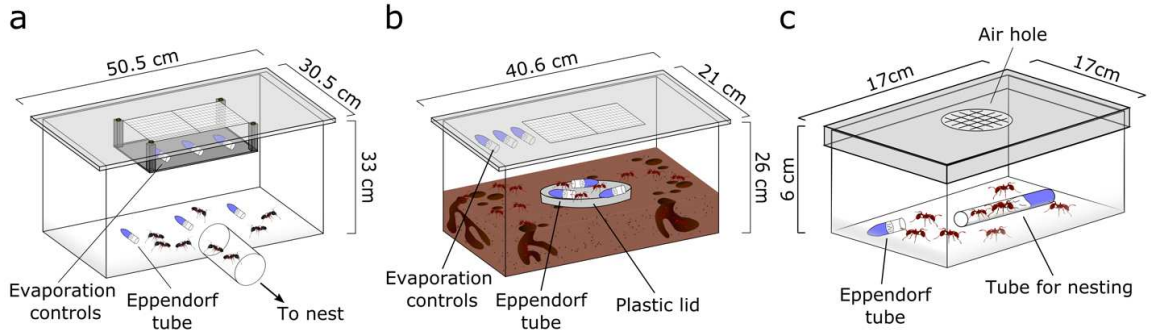

Figure 7.1. **Illustrations of experimental designs used to test effects of essential and non-essential amino acids on ant colony feeding preferences and reproductive fitness. (a, b) Experimental designs used to bioassay consumption of aqueous amino acid blends by carpenter ants, *Camponotus modoc,* and European fire ants, *Myrmica rubra.* Amino acid blends were presented in 1.5-mL Eppendorf tubes plugged with a piece of cotton wick to allow blend consumption by ants without spillage. For each *C. modoc* bioassay (a), Eppendorf tubes were presented in a container connected via Tygon tubing to the ants' nest; for each *M. rubra* bioassay (b), Eppendorf tubes were arranged on a plastic lid which was placed directly into the ants' nesting container. Evaporation control tubes were placed on a platform beneath the container lid (a) or taped directly to the underside of the lid (b). (c) Design used for testing the effect of essential and non-essential amino acids on *M. rubra* colony growth and worker mortality; small colonies, or groups of workers, were placed in Tupperware containers fitted with a test tube for nesting and an Eppendorf tube containing a nutrient blend. Note: drawings are not to scale.**

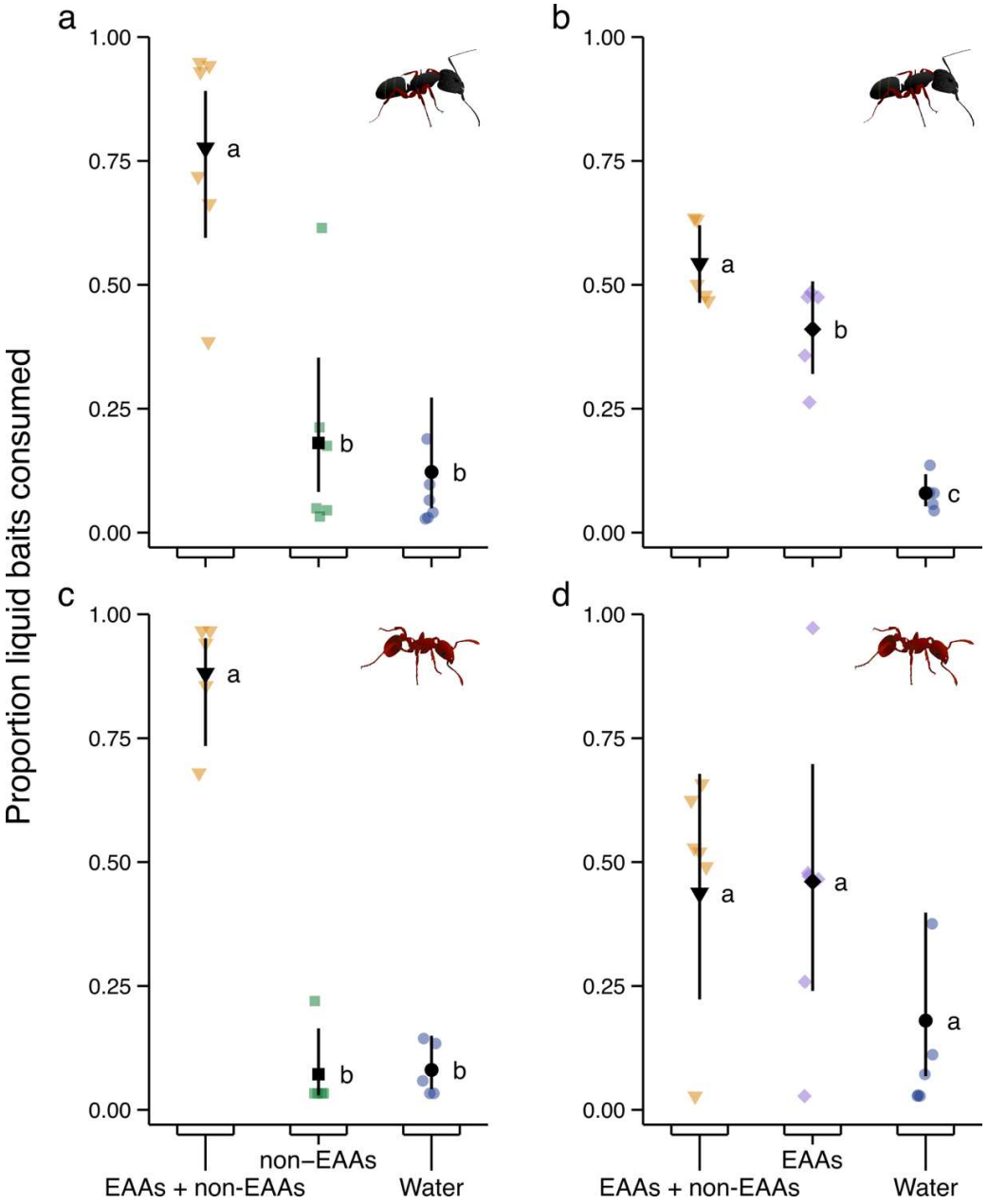

Figure 7.2. **Proportional consumption of aqueous amino acid baits by colonies of *C. modoc* [a (n = 6), b (n = 5)] and *M. rubra* [c (n = 5), d (n = 6)]. Colonies were offered aqueous baits of 11 essential amino acids (EAAs; 0.55% w/v), 10 non-essential amino acids (non-EAAs; 0.5% w/v), and both EAAs and non-EAAs (1.05% w/v) as well a water control. Each coloured symbol represents the result of an individual replicate, whereas black symbols and whiskers are modelled estimated marginal means and 95% confidence intervals from a GLMM. Treatment was a significant predictor of proportional consumption in all experiments except for experiment 4. In each subpanel, different letters assigned to proportional consumptions of amino acid baits denote statistically significant differences in Tukey adjusted pairwise comparisons (see Table D1).**

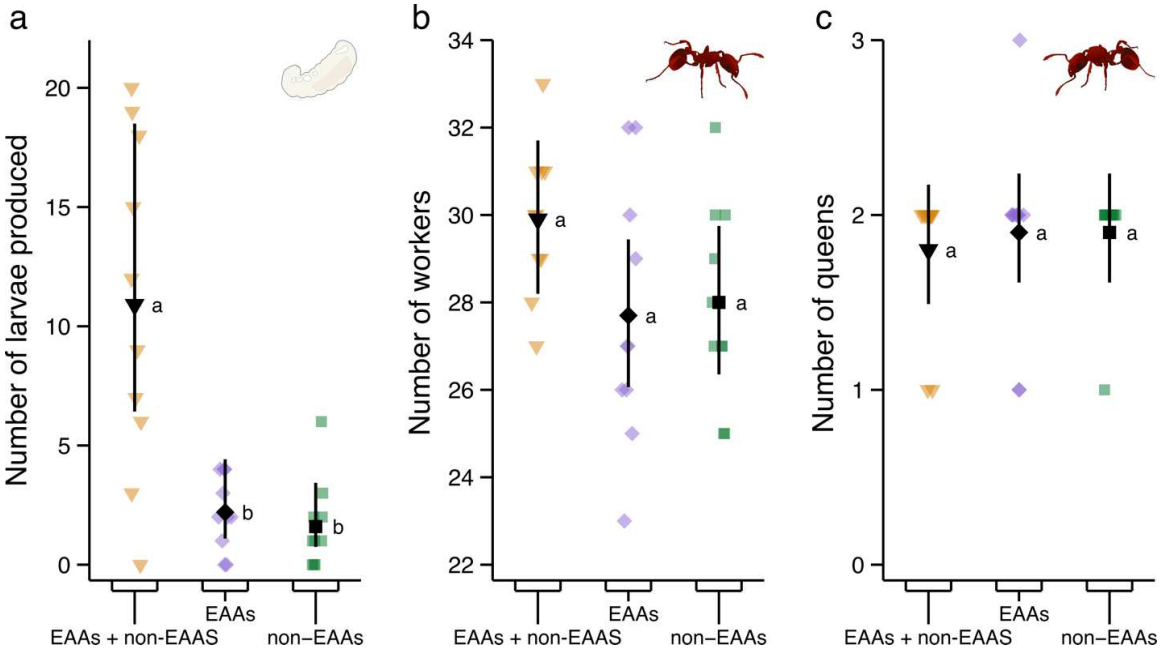

Figure 7.3. **Production of larvae, worker ants, and queen ants by *Myrmica rubra* colonies after a 16-week rearing experiment. Colonies were reared on synthetic diets containing 11 essential amino acids (EAAs, 1.1% w/v, Table 7.2), 10 non-essential amino acids (non-EAAs, 1.0% w/v, Table 7.2), and both EAAs and non-EAAs. Each coloured symbol represents the results of a single colony (n = 10), whereas black symbols and whiskers are modelled estimated marginal means and 95% confidence intervals from a GLMM. Treatment was a significant predictor for the number of larvae (likelihood ratio test: $p < 0.05$) but not for the number of worker and queen ants produced. In each subpanel, different letters assigned to numbers of larvae, worker and queen ants produced in response to amino acid bait composition denote statistically significant differences in Tukey adjusted pairwise comparisons (see Table D2).**

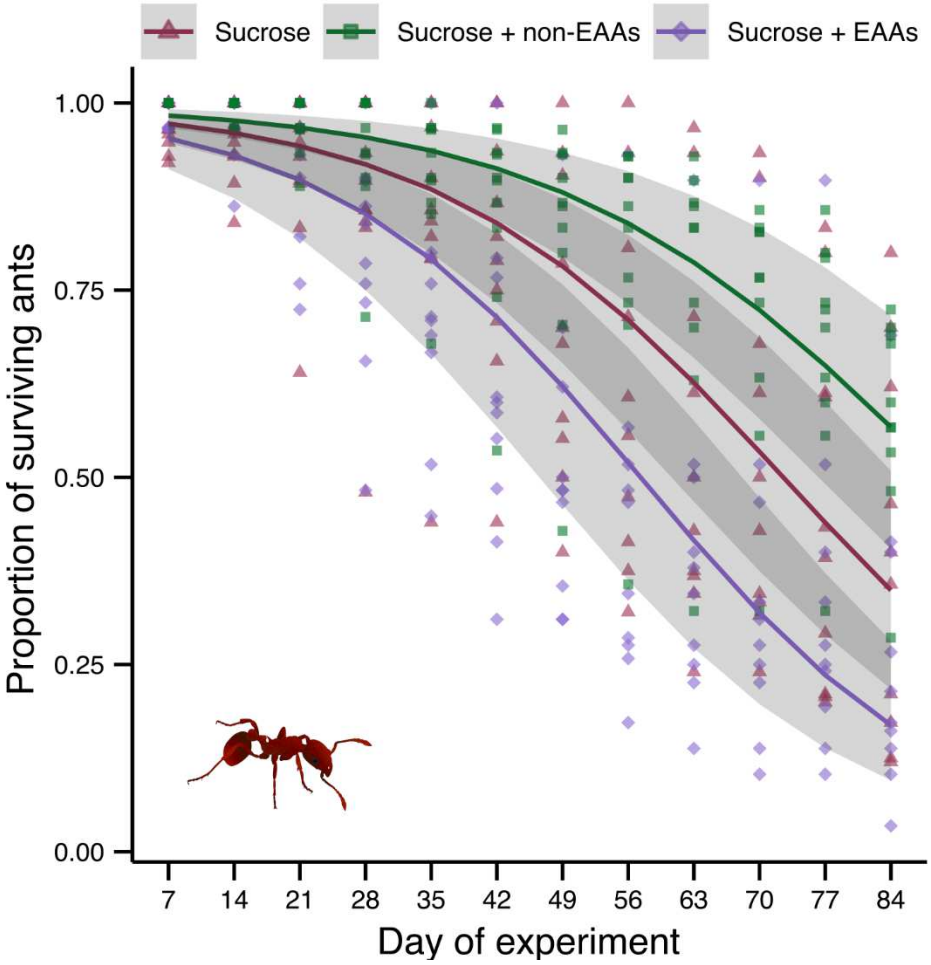

Figure 7.4. **Proportional survival of 30-worker groups of *Myrmica rubra* (n = 10) when fed aqueous solutions of sucrose only, sucrose plus 11 essential amino acids (EAAs; Table 7.2), and sucrose plus 10 non-essential amino acids (non-EAAs; Table 7.2). Coloured symbols represent the proportion of surviving workers in individual replicates. Lines and shaded regions are back transformed model predictions and 95% confidence intervals from a binomial GLMM with a logit link function. The type of nutrient solution, day of experiment, and interaction between nutrient solution and day were all significant predictors in our model (likelihood ratio test: $p < 0.05$).**

Chapter 8.

Effects of macro- and micro-nutrients on momentary and season-long feeding responses by select species of ants

A similar version of this chapter has been accepted: Renyard, A., Gooding, G., Chalissery J.M., Petrov, J., and Gries, G., (accepted). Effects of macro- and micro-nutrients on momentary and season-long feeding responses by select species of ants Sci. Rep. (Ref: Submission ID c84c743f-bad6-4dfc-90a1-06937db986c6)

8.1. Abstract

Few studies have investigated the relative contribution of specific nutrients to momentary and season-long foraging responses by ants. Using western carpenter ants, *Camponotus modoc,* and European fire ants, Myrmica *rubra,* as model species, we: (1) tested preferential consumption of various macro- and micro-nutrients; (2) compared consumption of preferred macro-nutrients; (3) investigated seasonal shifts (late May to mid-September) in nutrient preferences; and (4) tested whether nutrient preferences of *C. modoc* and *M. rubra* pertain to black garden ants, *Lasius niger,* and thatching ants, *Formica aserva*. In laboratory and field experiments, we measured nutrient consumption by weighing Eppendorf tubes containing aqueous nutrient solutions before and after feeding by ants. Laboratory colonies of *C. modoc* favored nitrogenous urea and essential amino acids (EAAs), whereas *M. rubra* colonies favored sucrose. Field colonies of *C. modoc* and *M. rubra* preferentially consumed EAAs and sucrose, respectively, with no sustained shift in preferred macro-nutrient over the course of the foraging season. The presence of a less preferred macro-nutrient in a nutrient blend did not diminish the blend's 'appeal' to foraging ants. Sucrose and EAAs singly and in combination were equally consumed by *L. niger,* whereas *F. aserva* preferred EAAs. Baits containing both sucrose and EAAs were consistently consumed by the ants studied in this project and should be considered for pest ant control.

8.2. Introduction

Adequate nutrition is vital for colony fitness in ants[1,2], affecting colony functioning, brood production and development, and worker survival[2]. The availability of carbohydrates as an energy source affects both the activity[3–6] and the longevity of worker ants[3,4,6–10], which, in turn, modulate foraging activities and aggressiveness of ant colonies[3–6,11,12]. Protein sources, in combination with carbohydrates, are essential for egg production by queens and brood development[3,4,13–16], but in high amounts are toxic to workers[7–10].

Ant colonies face challenges to meet their nutritional needs. Foragers must locate and recognize required nutrient resources, and integrate their own nutritional needs with those of their nestmates[2]. Ants locate food resources by responding to their odor plumes[17–24], or by following trail pheromone deposited by forager ants[25]. The pattern of nutrient collection by ants may shift over time, with more carbohydrate- or protein-rich resources collected at different times of year[26–29]. Shifts in nutrient preference may be caused by demographic shifts in ant colonies or shifts in nutrient availability in the ants' habitat. Experimentally increased amounts of colony brood mobilized foraging in *L. niger* workers[30], prompted more food and more protein collection by *Rhytidoponera* ants[31,32], and more protein consumption by *Ochetellus glaber* ants[33]. Ant colonies that have plenty of readily available nutrients preferentially seek foods containing scarcer nutrients[34,35].

Ants assess food quality through the presence and concentration of certain macro-nutrients (proteins, carbohydrates, lipids) and micro-nutrients (e.g., salts, vitamins). In aphid honeydew, ants recognize the different types of sugar molecules[36–41] (e.g., sucrose, melezitose) and generally they prefer resources with high sugar concentration[39,41,42]. Ants also require proteins, and obtain amino acids – the 'building blocks' of proteins – by e.g. (*i*) ingesting free amino acids from plant nectar[43–46], (*ii*) digesting proteins from insect prey[47], and (*iii*) by acquiring the amino acids that symbiotic gut microbes produce from nitrogenous waste[15,48]. Ants prefer essential to non-essential amino acids[49], and recognize when specific amino acids are lacking[49]. Ants also feed on certain oils[38], and recognize distinct fatty acids (e.g., oleic acid) and glycerides (e.g., 1,2-diolein) that are present on the surface of insect prey, deceased insects, and seed

elaiosomes, and that serve as pick-up cues by ants[50–53]. Furthermore, ants recognize and consume micro-nutrients, including salts[54–57] (e.g., NaCl) and some B-vitamins[58].

Very few studies have comprehensively examined the ants' preferences for specific macro- and micro-nutrients or for these nutrients as components of complex nutrient blends[37,49,58]. Although seasonal shifts in nutrient preference by ants have been demonstrated[26–29], these nutrients were often presented as complex blends (e.g., tuna, fruit conserves) with sometimes different physical properties, making it difficult to attribute the ants' preferential feeding responses to any one nutrient component.

Previously, we have determined nutrient preferences of two generalist ant species, the western carpenter ant, *Camponotus modoc,* and the European fire ant, *Myrmica rubra* [41]. *Camponotus modoc* inhabits temperate forests along the western coast of North America[59] and excavates nests in the wood of conifer trees[60]. *Myrmica rubra* is an aggressive soil-dwelling ant that is native to Eurasia but has invaded the east and west coasts of North America[61]. While both species prefer sucrose to other saccharides[41], and both species prefer essential to non-essential amino acids (AR, unpublished data), only *C. modoc* also consumes urea (AR, unpublished data). *Lasius niger* is a widespread[62,63], temperate, soil-dwelling ant that regularly tends aphids and prefers aphid-derived sugars such as melezitose to common sugars such as sucrose[36,64]. *Lasius niger* also preferentially feeds on diverse amino acid blends[64,65] but any potential preference for specific amino acids is not known. *Formica aserva* is a brood-raiding ant, nesting in woody debris[66] such as stumps[67]. It tends aphids[68] and collects insect prey[69] but nutrient preferences are not yet documented. We selected these four species to represent ants in diverse taxa with contrasting morphology and body size, life history traits, and habitat preferences.

Here, we investigated momentary (*ad hoc*), and seasonal, nutrient preferences of *C. modoc* and *M. rubra* colonies*,* and determined whether their observed nutrient preferences apply to other ant taxa. Specifically, we: (1) tested nutrient consumption within groups of either macro-nutrients or micro-nutrients; (2) compared consumption of preferred macro-nutrients; (3) investigated potential seasonal shifts in nutrient preferences; and (4) tested whether nutrient preferences of *C. modoc* and *M. rubra* pertain to *L. niger* and *F. aserva.*

8.3. Materials and methods

8.3.1. Maintenance of laboratory ant colonies

We collected and maintained nine colonies of *C. modoc* between 2016 and 2020, as reported[70]. Briefly, we excised infested log sections from coniferous forests near Squamish (British Columbia), and transferred these sections to large plastic bins (64 × 79 × 117 cm) kept in an outdoor, under-cover area of the Science Research Annex at Simon Fraser University. All colonies experienced natural weather and light cycles which can be important for colony survival[71]. Bins were connected to glass containers (30.5 × 26 × 50.8 cm) which served as the ants' foraging area. The upper inner bin and container walls were coated with an equal mix of Vaseline (Unilever, London, UK) and paraffin oil (Anachemia, Lachine, QC H8R1A3, CA) to prevent ant escape. Ants were provisioned with apples, deceased cockroaches, and 20% sugar water *ad libitum.* Containers and bins had mesh covered holes to allow air exchange.

We collected and reared invasive *M. rubra* similar to previous reports[41,72], with some modifications. In the summer of 2021, 10 colonies were dug up with their nesting soil at Inter-river Park (North Vancouver, BC, CA), and temporarily placed in glass jars (1L). Colonies were then transferred to separate glass containers (26 × 21 × 40.6 cm), with mesh-covered holes in container lids, and upper inner container walls coated with Vaseline and paraffin oil. Colonies were maintained indoors at 25–30 °C under a natural daylength cycle, and were provisioned with food as described above. The soil surface of containers served as the ants' foraging area. Every two weeks, water was added to the soil to ensure adequate moisture content. Colonies were kept indoors, instead of outdoors, to minimize the risk of ant escape on university campus.

8.3.2. Preparation of test stimuli

Prior to experiments, test stimuli were prepared by weighing nutrients (TR 204 scale; Denver Instrument Company, CO, USA; see Table 1 for number of test stimuli, and Table E1 for nutrient compositions) and mixing them into water, accounting for ~50% of the final volume. Once nutrients were dissolved, distilled water was added until the desired weight by volume solution (w/v) was reached. Aliquots (1 mL) of solutions were pipetted into 1.5-mL Eppendorf tubes (Thermo Fisher Scientific, Waltham, MA

02451, USA) and kept frozen until use in bioassays. For each experiment, we prepared as many tubes, including evaporation control tubes (see below), as required for testing stimuli consumption by all colonies. Sucrose and essential amino acids (EAAs) were selected as nutrients based on previous studies[41], and EAAs were assembled drawing on both an article[15] and personal communication with its senior author. Fatty acids (oleic, linoleic, linolenic) and glycerides (1,2 diolein, triolein) were tested because they serve as pickup cues for ants[50,52,73]. Selections of sterols, and their approximate ratio, were based on reported dietary needs of insects[47]. Salts and vitamins were tested at equal ratio drawing on the composition of a synthetic ant diet[74] (see Table E1 for compositions of test stimuli).

8.3.3. General protocol for laboratory bioassays

On any experimental day, test stimuli were removed from the freezer, thawed, and vortexed, thus ensuring that all solutes were dissolved. Eppendorf tubes were then stuffed with a 1-cm-long piece of a cotton dental wick (Richmond Dental & Medical, Charlotte, NC 28205, USA) to allow nutrient consumption by ants without spillage (Fig. 8.1a). For each test stimulus, two tubes were prepared: one for ant consumption and another for tracking passive water evaporation during bioassays. All tubes were weighed just prior to, and at the end of, bioassays. In each bioassay replicate, we tested the consumption response of a different colony and prepared as many Eppendorf tubes as stimuli were tested (typically 4–5), with each tube containing a specific nutrient solution or a plain water control. Prior to the onset of a bioassay, tubes were weighed, uncapped and presented to ants, allowing them to forage for 4–6 h (see below). Bioassay times for *C. modoc* and *M. rubra* colonies were set to 4 h and 6 h, respectively, accounting for differences in worker size[60,61] and considering the time that was needed to obtain measurable consumption rates in preliminary experiments. Colonies for laboratory experiments were randomly selected on each experimental day, and were given at least 36 h between experiments. Each colony experienced a particular set of test stimuli only once. All laboratory experiments were conducted between June and early September.

Bioassays with *C. modoc* (and *M. rubra* below) were run mostly on warm and sunny days when colonies are most active (AR, pers. obs.). Prior to bioassays, colonies were deprived of cockroaches and apples for 24 h, and of sugar water for 4 h (the maximum time elapsed before ants attempted to chew out of their containers).

Bioassays were run in plexiglass containers (50.5 × 30.5 × 33 cm) covered by lids with mesh holes to allow ventilation (Fig. 8.1b). Tubes were taped, with positions randomly assigned and spaced equidistantly in an arc, to the container bottom 22 cm away from the container entrance hole. Corresponding evaporation control tubes were taped to a plexiglass platform suspended from the container lid. Just prior to initiating a bioassay, all tubes were uncapped and each container was connected via Tygon® tubing (diam.: 2.54 cm) and barbed plumbing connectors (diam.: 2.54 cm) to a *C. modoc* housing bin, allowing ants to freely forage in a container. Bioassay replicates were run for 4 h but were terminated sooner if ants had completely consumed the test solution of any one tube. Bioassay containers were cleaned with hexane and ethanol (70%), and plumbing fixtures and Tygon® tubings were washed with soapy water.

For bioassays with *M. rubra,* nest containers were co-opted as bioassay containers, and colonies were food-deprived for 24 h to motivate foraging. For each replicate, Eppendorf tubes were taped, randomly assigned, to the edge of a jar lid (diam: 15 cm), and corresponding evaporation control tubes were taped, inaccessible to ants, to the underside of container lids. Bioassay replicates were initiated by uncapping all Eppendorf tubes, and placing jar lids with Eppendorf tubes on the soil surface inside bioassay containers. Bioassays were run for 6 h but were terminated sooner if ants had consumed the entire test solution in an Eppendorf tube. Between replicates, jar lids were washed with soapy water.

8.3.4. Protocol of field experiment

In preparation for field experiments, Eppendorf tubes with nutrient solutions for ant consumption, and evaporation control tubes, were thawed, weighed, and then transported to the field in a cooler. Tubes were spaced around the entrance of ant nests or next to ant foraging trails, with tube positions randomly assigned in each replicate. For *C. modoc,* Eppendorf tubes were affixed 5 cm apart to trees or logs housing a *C. modoc* nest (Fig. 8.1c). For *L. niger,* we located nests at the base of trees, and affixed tubes next to each other on the trunk of trees alongside the ants' foraging trails. For *M. rubra,* tubes were placed 5 cm apart around the entrance of subterranean nests. For *F. aserva*, tubes were placed on top of tree stumps that contained an ant nest. For all field studies, evaporation control tubes were placed in Tupperware containers (15 × 9 × 10 cm) with a mesh-covered hole in the lid, and containers were set near ant nests. Replicates with

colonies of *M. rubra* (n = 10; repeated on 7 dates)*, L. niger* (n = 10)*, C. modoc* (n = 13; repeated on 6 dates)*,* and *F. aserva* (n = 10) were run for 4 h, 16 h, 24 h, and 24 h, respectively. Bioassays were run between 10:00-14:00 for *M. rubra,* 17:00–09:00 for *L. niger*, and between 11:00–11:00 for both *C. modoc* and *F. aserva.* Experimental time periods for each species were set according to time periods needed in preliminary studies to obtain measurable consumption rates. After replicates were terminated, tubes were capped, transported to the laboratory in a cooler, and weighed. All 4- to 24-h field studies were run on warm and sunny days with observable ant activity. Experiments with *C. modoc* and *M. rubra* were run from late May to mid-September, and experiments with *L. niger* and *F. aserva* were run in June and August, respectively.

8.3.5. Specific Experiments

Assessing consumption of various macro- and micro-nutrients (Exps. 1–12; Lab)

In experiments 1–2 (Table 1), we offered colonies of *C. modoc* and *M. rubra* a choice between aqueous sucrose solutions at three concentrations (0.625%, 1.25%, and 2.5% w/v) and a water control.

Experiments 3–8 (Table 1) tested consumption of lipid-related nutrients by *C. modoc* and *M. rubra* colonies. Each of three lipid types (glycerides, fatty acids, sterols) consisted of 2–4 constituents (Table E1) which were formulated in an aqueous solution at two concentrations (Exps. 3–4: glycerides: 0.5%, 1.0% w/v; Exps. 5–6: fatty acids: 1.25%, 2.5% w/v; Exps. 7–8: sterols: 0.5%, 1.0% w/v), using Tween 80 as the emulsifier. In each experiment, both Tween 80 in water, and water, served as control stimuli.

Experiment 9–12 (Table 1) tested consumption of micro-nutrient salts or vitamins by *C. modoc* and *M. rubra* colonies. Each type of micro-nutrient consisted of 7–11 constituents (Table E1) which were dissolved in water at three concentrations (Exps. 9–10: salts: 0.25%, 0.5%, 1.0% w/v; Exps. 11–12: vitamins: 0.25%, 0.5%, 1.0% w/v), with water serving as the control stimulus in each experiment.

Comparing consumption of preferred macro-nutrients (Exps. 13–20; Lab)

Experiments 13–20 (Table 1) compared consumption of macro-nutrients that *C. modoc* or *M. rubra* colonies were previously shown to preferentially consume, including

urea (AR, unpublished data), essential amino acids (EAAs; AR, unpublished data), and sucrose[41]. In experiments 13–16, aqueous solutions of urea, EAAs, and sucrose were tested singly and in ternary combination, with plain water as the control stimulus. Single components were tested at the same 'unadjusted' concentration as in the ternary blend (urea 2.5%; EAAs 0.55%; sucrose 2.5% w/v) or at an 'adjusted' concentration (5.55% w/v) that equalled the total concentration of the ternary blend (5.55%). Each component in the ternary blend was tested at the lowest concentration found effective in pre-screening experiments (see Result of Exps. 1–12; AR, unpublished data). In experiments 17–20 (Table 1), aqueous solutions of urea, EAAs, and sucrose were tested in all binary and ternary combinations, again with plain water as the control stimulus. Binary combinations were tested at the same 'unadjusted' concentration as in the ternary blend (urea [2.5 %] + EAA [0.55 %]; urea [2.5%] + sucrose [2.5%]; EAA [0.55%] + sucrose [2.5%]) or at an 'adjusted' concentration (urea [4.55 %] + EAA [1.0 %]; urea [2.775%] + sucrose [2.775%]; EAA [1.0%] + sucrose [4.55%]) that equalled the total concentration of the ternary blend (5.55%).

Evaluating potential seasonal shifts in nutrient consumption (Exps. 21–22; Field)

Experiments 21–22 (Table 1) investigated potential seasonal shifts in nutrient preferences exhibited by field colonies of ants. We worked with 13 colonies of *C. modoc* and 10 colonies of *M. rubra* located along the Mamquam forest service road (near Squamish, BC, Canada) and at Inter River Park (District of North Vancouver, BC, Canada), respectively. Drawing on results of preceding experiments that both *C. modoc* and *M. rubra* had preferentially consumed the 'adjusted' binary blend of EAA + sucrose (see Results), we offered each ant colony four Eppendorf tubes that contained: (1) EAA (5.55%); (2) sucrose (5.55%), (3) EAA (1.0%) + sucrose (4.55%); and (4) plain water (control). Throughout the summer season, we measured nutrient consumption by colonies in circa 3-week intervals on six dates for *C. modoc* colonies (18 June 2021 to 07 September 2021), and on seven dates for *M. rubra* colonies (21 May 2021 to 13 September 2021).

Investigating nutrient consumption of L. niger and F. aserva (Exps. 23–24; Field)

We worked with 10 field colonies each of *L. niger* and *F. aserva* that were located on the Burnaby campus of Simon Fraser University and along the Mamquam forest

service road (see above), respectively. Each nest was offered four Eppendorf tubes that contained: (1) EAAs (5.55%); (2) sucrose (5.55%), (3) EAAs (1.0%) + sucrose (4.55%); and (4) plain water (control).

8.3.6. Statistical analyses

To calculate the amount of each nutrient solution that was consumed by a colony, we first determined the weight loss of the corresponding evaporation control solution, and then subtracted this value from the weight loss of the test solution. To account for differences in colony size and foraging activity between colonies, we analysed proportions, rather than absolute amounts, of nutrient solutions consumed. To obtain proportional consumption data for a colony in any experimental replicate, we divided the amount (weight) of each nutrient solution consumed by the total amount of all nutrient solutions consumed. When there had been little feeding activity by a colony, some consumption data became less than zero (~ -7 mg) following weight loss subtraction due to water evaporation measured in evaporation controls (see above). As evaporation control tubes were close to the arena vent (Fig. 8.1b), these small negative values could be due to slightly elevated rates of evaporation. As there could not be 'negative feeding' on a nutrient solution by a colony, we considered these less-than-zero values to be zero. We included replicates in data analyses when a colony had positive consumption responses for at least two of all the nutrient solutions that were tested in that replicate. Using a beta distributed generalized linear mixed model (GLMM), we applied a standard transformation to restrict our data between the bounded interval of 0 and 1. In experiments 1–20 and 23–24, we fit proportion consumed as our response variable and treatment as our predictor, with ant colony as a random intercept. For experiments 21–22, we fit proportion consumed as our response variable and treatment, date, and interaction between treatment and date as predictors, with ant colony as a random intercept. We evaluated the significance of predictors, using likelihood ratio tests and made Tukey adjusted pairwise comparisons between mean proportional consumption values between treatments.

Data[75] were analysed and graphed using R (v. 4.2.2) and R studio (v. 2022.07.1+554)[76]. Data were processed using functions from the tidyverse[77] and the plyr package[78]. GLMMs were fit using the glmmTMB package[79], and model fit, residual normality, variance, and over/under dispersion patterns were inspected, using the

DHARMa package[80]. We obtained estimated marginal means and 95% confidence intervals using the emmeans package[81]. We produced graphics using the ggplot2 package[82] and completed figure assemblies in Inkscape (v. 1.0.2).

8.4. Results

8.4.1. Assessing consumption of various macro- and micro-nutrients (Exps. 1–12; Lab)

Concentrations of sucrose in aqueous solutions affected their consumption by *C. modoc* colonies ($\chi^2 = 8.75$, d. f. = 3, $p = 0.03$; Figure E1a) and by *M. rubra* colonies ($\chi^2 = 54.18$, d. f. = 3, $p < 0.0001$; Figure E1b). Colonies of *C. modoc* preferentially consumed the 1.5% (w/v) sucrose solution, which was the only solution consumed significantly more than water. Colonies of *M. rubra* consumed significantly more of the 2.5% (w/v) sucrose solution than of any other solution including the water control (Table E2).

Glycerides in emulsified aqueous solutions did not prompt consumption by *C. modoc* colonies ($\chi^2 = 2.03$, d. f. = 3, $p = 0.57$; Figure E2a) and *M. rubra* colonies ($\chi^2 = 0.72$, d. f. = 3, $p = 0.87$; Figure E2b, Table E3).

Fatty acids in emulsified aqueous solutions significantly affected feeding responses of *C. modoc* colonies ($\chi^2 = 8.46$, d. f. = 3, $p = 0.04$; Figure E2c) and *M. rubra* colonies ($\chi^2 = 8.90$, d. f. = 3, $p = 0.03$; Figure E2d). Increasing concentrations of fatty acids lowered consumption, with the 2.5% solution being consumed the least (Table E3).

Sterols in aqueous solutions did not affect consumption by *C. modoc* colonies ($\chi^2 = 2.47$, d. f. = 3, $p = 0.48$; Figure E2e) but did affect consumption by *M. rubra* colonies ($\chi^2 = 9.90$, d. f. = 3, $p = 0.02$; Figure E2f). Colonies of *M. rubra* consumed more of the 0.5% sterol solution than of the 1% sterol solution and the water control but not of the tween + water control (Table E3). The 1% sterol solution, Tween + water, and water all prompted comparable consumption (Table E3).

Salts in aqueous solutions did not affect consumption by *C. modoc* colonies ($\chi^2 = 0.89$, d. f. = 3, $p = 0.83$; Figure E3a) but was a significant predictor of proportional consumption by *M. rubra* colonies ($\chi^2 = 32.26$, d. f. = 3, $p < 0.0001$; Figure E3b).

Increasing salt concentrations lowered consumption, with the 1% solution being consumed the least (Table E3).

Vitamins in aqueous solutions did not affect consumption by *C. modoc* colonies (χ^2 = 0.10 d. f. = 3, p = 0.99; Figure E3c) but affected consumption by *M. rubra* colonies (χ^2 = 20.66, d. f. = 3, p <0.001; Figure E3d). Colonies of *M. rubra* equally consumed the 1% vitamin solution and plain water, both of which more than the 0.25% and 0.5% solution (Table E4).

8.4.2. Comparing consumption of preferred macro-nutrients (Exps. 13–20; Lab)

Colonies of *C. modoc* and *M. rubra* differentially consumed 1- and 3-component aqueous solutions of urea, EAAs, and sucrose, and plain water (control stimulus) (Table 1) (*C. modoc:* unadjusted concentrations of nutrients in aqueous solutions: χ^2 = 71.15, d. f. = 4, p <0.0001, Fig. 8.2a; *C. modoc*: adjusted concentrations: χ^2 = 57.72, d. f. = 4, p <0.0001, Fig. 8.2b; *M. rubra*: unadjusted concentrations: χ^2 = 61.59, d. f. = 4, p <0.0001, Fig. 8.2c; *M. rubra*: adjusted concentrations: χ^2 = 91.7, d. f. = 4, p < 0.0001, Fig. 8.2d). *Camponotus modoc* colonies preferentially consumed solutions containing urea, EAAs, or both (together with sucrose) (Fig. 8.2a,b). At adjusted nutrient concentrations, EAAs on their own and in ternary combination with urea and sucrose were most heavily consumed (Table E5). When nutrient concentrations were unadjusted, *M. rubra* colonies preferentially consumed sucrose, and sucrose in ternary combination with urea and EAAs. At adjusted nutrient concentrations, *M. rubra* colonies preferentially consumed single-nutrient solutions of EAAs and sucrose, followed by the ternary blend of EAAs, sucrose, and urea (Table E5).

There was also differential consumption of macro-nutrients by *C. modoc* and *M. rubra* colonies when sucrose, EAAs, and urea were offered – at unadjusted and adjusted nutrient concentrations – in all possible binary and ternary combinations, along with plain water as the control stimulus (*C. modoc*: unadjusted concentrations: χ^2 = 30.79, d. f. = 4, p < 0.0001, Fig. 8.3a; *C. modoc*: adjusted concentrations: χ^2 = 30.71, d. f. = 4, p <0.0001, Fig. 8.3b; *M. rubra*: unadjusted concentrations: χ^2 = 61.329, d. f. = 4, p < 0.0001, Fig. 8.3c; *M. rubra*: adjusted concentrations: χ^2 = 115.38, d. f. = 4, p < 0.0001, Fig. 8.3d). At unadjusted nutrient concentrations, *C. modoc* colonies equally consumed

all binary and ternary nutrient blends, significantly preferring all of them to plain water (Fig. 8.3a; Table E6). At adjusted nutrient concentrations, *C. modoc* colonies consumed the blend of urea + EAAs significantly more than the blend of urea + sucrose, and water, but not significantly more than the blend of EAAs + sucrose, and the ternary blend (Fig. 8.3b; Table E6). At unadjusted nutrient concentrations, *M. rubra* colonies preferentially consumed the blend of EAAs + sucrose and the ternary blend, followed by blends of urea + sucrose and urea + EAAs, with the latter blend being consumed as little as water (Fig. 8.3c; Table E6). At adjusted nutrient concentrations, the blend of EAAs + sucrose was most heavily consumed, followed by the ternary blend, and by binary blends of urea + sucrose and urea + EAAs, which had similar levels of consumption, both significantly higher than water (Fig. 8.3d; Table E6).

8.4.3. Evaluating potential seasonal shifts in nutrient consumption (Exps. 21–22; Field)

In the field experiment with *C. modoc* colonies, bait nutrient(s), date, and interaction between bait nutrient(s) and date, were all significant predictors of bait consumption by ants (bait nutrient(s): $\chi^2 = 371.1$, d. f. = 18, $p < 0.0001$; date: $\chi^2 = 117.04$, d. f. = 20, $p < 0.0001$; interaction between bait nutrient(s) and date: $\chi^2 = 109.95$, d. f. = 15, $p < 0.0001$; Fig. 8.4a). Invariably over time, *C. modoc* colonies preferentially consumed EAAs, and EAAs + sucrose in a binary blend (Fig. 8.4a; Table E7). Consumption of water, and of sugar, decreased over time[75] (Fig. 8.4a).

Similarly, in the field experiment with *M. rubra* colonies, bait nutrient(s), date, and interaction between bait nutrient(s) and date, were all significant predictors of bait consumption by ants [bait nutrient(s): $\chi^2 = 403.59$, d. f. = 21, $p < 0.0001$; date: $\chi^2 = 267.51$, d. f. = 24, $p < 0.0001$; interaction between bait nutrient(s) and date: $\chi^2 = 245.17$, d. f.= 18, $p < 0.0001$; Fig. 8.4b]. Across sampling dates, *M. rubra* colonies generally consumed more sucrose, and more sucrose + EAAs in a binary blend, than EAAs and water (Fig. 8.4b; Table E7). Sucrose consumption declined over time, whereas the consumption of sucrose in a binary blend with EAAs increased during the last three sampling dates[75] (Fig. 8.4b).

8.4.4. Investigating nutrient consumption of *L. niger* and *F. aserva* (Exps. 23–24; Field)

Bait nutrients affected bait consumption by *L. niger* colonies (χ^2 = 48.33, d. f. = 3, p < 0.0001; Fig. 8.5a) and *F. aserva* colonies (χ^2 = 12.29, d. f. = 3, p = 0.006; Fig. 8.5b). Colonies of *L. niger* equally consumed baits containing EAAs, sucrose, and EAAs + sucrose, all of which being preferred to plain water (control stimulus) (Table E7). Colonies of *F. aserva* preferentially consumed baits containing EAAs, which they consumed more than sucrose baits but (statistically) not more than EAA + sucrose baits (Table E7).

8.5. Discussion

Macronutrient preferences differed among the ant taxa we tested in our study which varied in size, life history, and preferred habitat. *Camponotus modoc* and *M. rubra* fed on macro-nutrients but not on micro-nutrients, and preferentially consumed specific macro-nutrients such as essential amino acids (EAAs) and sucrose. Each species, however, preferred a different macro-nutrient. *Camponotus modoc* favored nitrogenous urea and EAAs, whereas *M. rubra* favored sucrose. In contrast, neither species consumed micro-nutrients such as salts and vitamins, and lipid-related compounds such as glycerides and fatty acids. There was no shift in preferred macro-nutrient(s) over the course of the foraging season. Colonies of *C. modoc* preferentially and consistently consumed EAAs, and EEAs blended with sucrose, whereas *M. rubra* colonies generally consumed sucrose, and sucrose blended with EAAs. Macro-nutrients preferentially consumed by *C. modoc* and *M. rubra* were also readily consumed by *L. niger* and *F. aserva.* We did not observe other ant species at baits during field trials, indicating that all data were generated exclusively by our study species. Although macro-nutrient preference and consumption differed among species, the underlying drivers for these differences, such as contrasting life-history and spatiotemporal food availability, are yet to be studied.

Various species of ants distinguish between different saccharides[37–41], and between different amino acids[49]. To date, only *Camponotus* ants have been demonstrated to consume urea (this study; [83–85]). Lipid-related compounds such as glycerides and sterols were as unappealing as water controls, and fatty acids were

ingested even less than water. These results were not expected considering that ants use glycerides and fatty acids on the surface of food items as pickup cues[50–53]. Additionally, ants ingest oils[38], although it is not known how they distinguish between oil types. The propensity of ants to salt-feed increases with distance from the ocean[54], and is generally common in arboreal and herbivorous species[54,57 but see 56]. However, *C. modoc* did not consume salt solutions and *M. rubra* was deterred by them. This may have been due, in part, to the salt composition we tested for consumption by ants. We offered a blend of salts drawing on the composition of a synthetic ant diet[74], whereas other studies offered sodium chloride (NaCl) as a single salt. Vitamins did not elicit foraging responses in our study but B vitamin added to water improved its acceptance by the imported fire ant *Solenopsis richerti*, albeit to a lesser extent than sugars or amino acids[58]. The effects of certain vitamins on ant colony health remain inconsistent[14,86].

Foraging preferences by ants in our study were largely driven by the specific macro-nutrient that each ant favored. Colonies of *C. modoc* and *F. aserva* preferentially foraged on the nitrogenous macro-nutrients, urea and/or EAAs, whereas *M. rubra* colonies preferentially consumed sucrose. Based on mean feeding responses, field colonies of *L. niger* consumed sucrose and EEAs equally. However, feeding preferences of individual colonies observably differed, with some colonies favoring sucrose and others EAAs[75]. These data indicate that the colonies' preferred macro-nutrient may shift over time in accordance with the colonies' demographics and/or resource availability or competition in their habitat. Similarly, bait selectivity by tropical ants was affected by prior feeding experience and by competition with ant community members at bait stations[37]. In contrast, field colonies of *C. modoc* and *M. rubra* consistently favored EAAs and sucrose, respectively, demonstrating persistent selection of a specific macro-nutrient. Similarly, tropical ants species preferred specific, and contrasting, blends of sucrose and particular amino acids[37]. Geometric framework studies (investigating the effects of nutrient mixtures on ant health) concluded that ants prioritise sustained carbohydrate supplies [2] which are deemed essential for colony health[3,4,6–10], whereas field studies revealed that many ant species prefer blends of sucrose and amino acids to sucrose alone[37]. In our season-long (21 May to 13 September) field study with *C. modoc* and *M. rubra* (Fig. 8.4), the blend of sucrose + EAAs was consistently consumed at a level comparable to consumption levels of EAAs or sucrose alone. Also, there was no sustained temporal shift in preferred macro-nutrient or blend of macro-nutrients,

contrasting with previous reports that ants selectively seek carbohydrates or proteins at certain times during the foraging season[26–29].

Both intrinsic and extrinsic factors affect foraging preferences in ants. As an intrinsic factor, the presence of brood motivates food and protein collections[31–33]. However, this intrinsic factor did not seem to have affected seasonal foraging patterns of *C. modoc* and *M. rubra* colonies (Fig. 8.4). Colonies of both species have seasonal egg production peaks, but larvae are consistently present in nests[60,87], suggesting that fluctuations in the number of larvae may be too subtle to affect seasonal foraging activities. As an extrinsic factor, resource availability in time and space affects foraging patterns of ants, with scarce nutrients most intensely sought[34,35]. Seasonally, yellow crazy ants, *Anoplolepis gracilipes*, preferentially seek sugar-rich food in the wet season and protein-rich food in the dry season, despite brood being present in nests year round[27]. These preferences align with the shortages of protein-rich invertebrates during the dry season and sugar-rich honeydew from scale insects during the wet season[27]. Nutrient preferences may also differ according to the strata occupied by ants in an ecosystem[28,88–90]. For example, across six tropical biomes in South America, arboreal ants foraged most intensely on carbohydrates, whereas ground-nesting ants preferentially foraged on lipids[88]. Consistent preference by *C. modoc* for EAAs, and by *M. rubra* for sucrose, could imply that these resources are consistently limited and therefore are preferentially sought. That *M. rubra* equally consumed EAAs and sucrose in a laboratory experiment (Fig. 8.2d) but favoured sucrose, and sucrose blended with EEAs, in the season-long field experiment (Figs. 4b) could have been due to contrasting nutrients obtainable by laboratory and field colonies. In ants, both intrinsic and extrinsic factors are likely at play, simultaneously, although these interactions have not yet been explored.

Lastly, findings in our study have significant implications for control of (invasive) pest ants. Presently, leading commercial baits appeal to ‘sweet-loving ants’ but not to species that preferentially seek protein-rich food, and thus would require baits containing essential amino acids. In our study, colonies of *C. modoc* that preferentially ingested aqueous solutions of EAAs, and colonies of *M. rubra* that preferentially ingested aqueous solutions of sucrose, all consumed baits containing both EAAs and sucrose to the same extent as they consumed baits containing only their preferred macro-nutrient, indicating that the presence of a less preferred macro-nutrient as a bait constituent did

not diminish the bait's 'appeal'. The same conclusion applies to the other ant taxa tested in this study, *L. niger* and *F. aserva*. It follows that both sucrose and EAAs could be constituents in the same bait, and thus would be appealing to both 'sugar- and protein-loving ants'. Moreover, even if there were pest ants that shift their macro-nutrient preference over the foraging season from carbohydrates to proteins, or *vice versa*, both macro-nutrients would be present in the bait, thus retaining its season-long appeal to foraging ants. We favor boric acid as the lethal constituent in such a bait because – like sucrose and EAAs – it is water-soluble and once dissolved in water expresses antimicrobial activity[91,92], thus preventing spoilage of the bait's macro-nutrients.

8.6. Data Availability Statement

Data are available from Mendeley Data and can be accessed at: DOI: 10.17632/zbtvkyzwcj.1[75]

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8.9. Tables

Table 8.1. Research objectives (O), stimuli tested, numbers of replicates (n) run, and ant species bioassayed in experiments 1–24.

Exp. #	Stimuli[a] tested	n (species tested)
(O1) Assess consumption of various macro- and micro-nutrients		
1–2	Water *vs* 0.625%[b] *vs* 1.25% *vs* 2.5% sucrose	9 (*C. modoc*); 10 (*M. rubra*)
3–4	Water *vs* Tween 80 (0.05%) *vs* 0.5% glycerides *vs* 1.0% glycerides	9 (*C. modoc*); 9 (*M. rubra*)
5–6	Water *vs* Tween 80 (0.05%) *vs* 1.25% fatty acids *vs* 2.5% fatty acids	7 (*C. modoc*); 9 (*M. rubra*)
7–8	Water *vs* Tween 80 (0.05%) *vs* 0.5% sterols *vs* 1.0% sterols	7 (*C. modoc*); 9 (*M. rubra*)
9–10	Water *vs* 0.25% *vs* 0.5% *vs* 1.0% salts	8 (*C. modoc*); 9 (*M. rubra*)
11–12	Water *vs* 0.25% *vs* 0.5% *vs* 1.0% vitamins	8 (*C. modoc*); 10 (*M. rubra*)
(O2) Compare consumption between preferred macronutrients		
13 &15	Water *vs* urea (2.5%) *vs* EAAs (0.55%) *vs* sucrose (2.5%) *vs* blend (5.55%); unadjusted[c]	8 (*C. modoc*); 9 (*M. rubra*)
14 & 16	Water vs urea (5.55%) *vs* EAAs (5.55%) *vs* sucrose (5.55%) *vs* blend (5.55%); adjusted[c]	9 (*C. modoc*); 8 (*M. rubra*)
17 & 19	Water vs urea + EAAs (2.5%; 0.55%) *vs* urea + sucrose (2.5%; 2.5%) *vs* EAAs + sucrose (0.55; 2.5%) *vs* blend (5.55%); unadjusted	9 (*C. modoc*); 9 (*M. rubra*)
18 & 20	Water vs urea + EAAs (4.55%; 1.0%) *vs* urea + sucrose (2.775%; 2.775%) *vs* EAAs (1.0%) + sucrose (4.55%) *vs* blend (5.55%); adjusted	9 (*C. modoc*); 9 (*M. rubra*)
(O3) Evaluate seasonal nutrient preferences		
21–22	Water vs EAAs (5.55%) vs sucrose (5.55%) vs blend (5.55%)	12–13 (*C. modoc*); 10 (*M. rubra*)
(O4) Investigate preferences of other ant taxa		
23–24	Water *vs* EAAs (5.55%) *vs* sucrose (5.55%) *vs* blend (5.55%)	10 (*L. niger*); 10 (*F. aserva*)

a For detailed stimulus compositions and suppliers of nutrients see Table E1

b Percentages are expressed as weight by volume (w/v)

c 'Unadjusted' and 'adjusted' refer to solutions containing one or two types of macro-nutrients, each type with the same percentage concentration as in the ternary blend ('unadjusted blend'), or with its concentration increased to match the total concentration of the ternary blend ('adjusted blend').

8.10. Figures

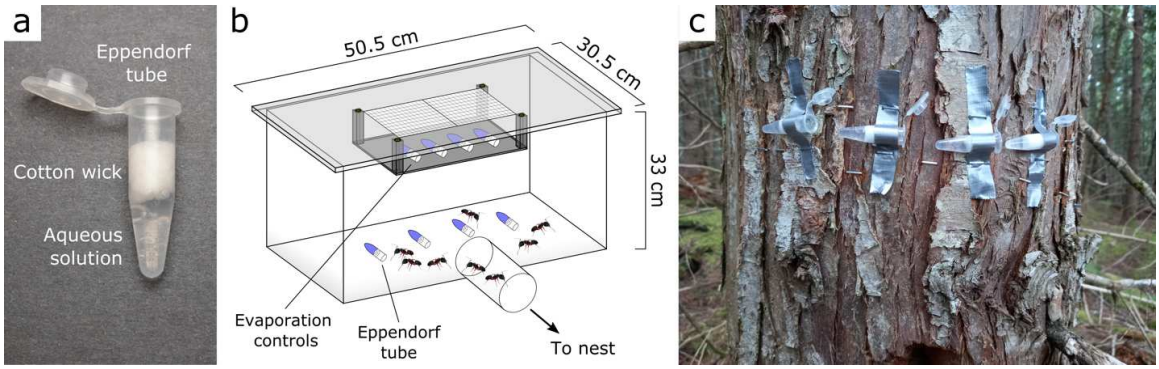

Figure 8.1. **Design of laboratory and field experiments for testing comparative food consumption by ants. (a) Photograph of an Eppendorf tube containing an aqueous nutrient solution retained by a piece of dental cotton wick, enabling ants to consume the liquid bait without spillage. (b) Bioassay container for carpenter ants, *Camponotus modoc,* fitted with 1.5-mL Eppendorf tubes containing aqueous nutrient solutions. Similar methodology was used for testing food consumption by European fire ants, *Myrmica rubra*[41]. Evaporation control tubes were placed on a small platform suspended from the container ceiling. (c) Eppendorf tubes affixed to a tree to test for preferential food consumption by *C. modoc* and black garden ants, *Lasius niger.* For field experiments with *M. rubra* and *Formica aserva,* Eppendorf tubes were placed on the ground.**

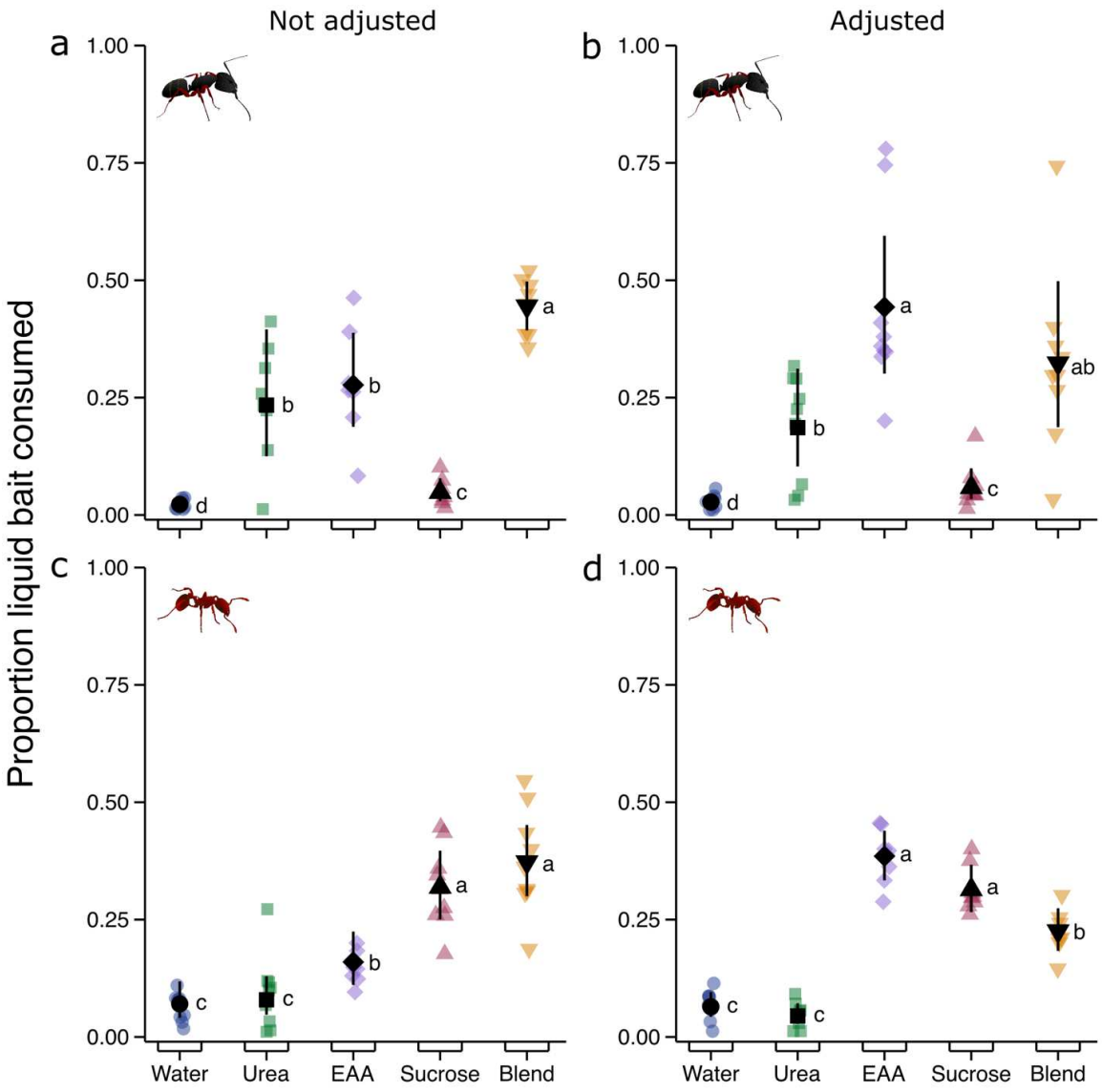

Figure 8.2. **Comparative consumption of liquid food baits by colonies of *Camponotus modoc* carpenter ants [a (n = 8), b (n = 9)], and *Myrmica rubra* fire ants [c (n = 9), d (n = 8)]. Colonies were offered a choice of aqueous solutions of macro-nutrients – urea, essential amino acids (EAAs), and sucrose – that were presented singly and in a ternary blend ('Blend'). The concentration of single macro-nutrients was either not adjusted [a, c; urea (2.5%); EAAs (0.55 %); sucrose (2.5%) w/v] or adjusted (b, d; urea, EAAs, and sucrose all 5.55%) to the same total concentration as the ternary blend [urea (2.5%), EAAs (0.55 %), and sucrose (2.5%) w/v]. Coloured symbols indicate consumption rates of individual colonies (replicates) and black symbols represent modelled estimated marginal means and 95% confidence intervals. Means with different letters are statistically different ($p < 0.05$) in pairwise comparisons (see Table E5).**

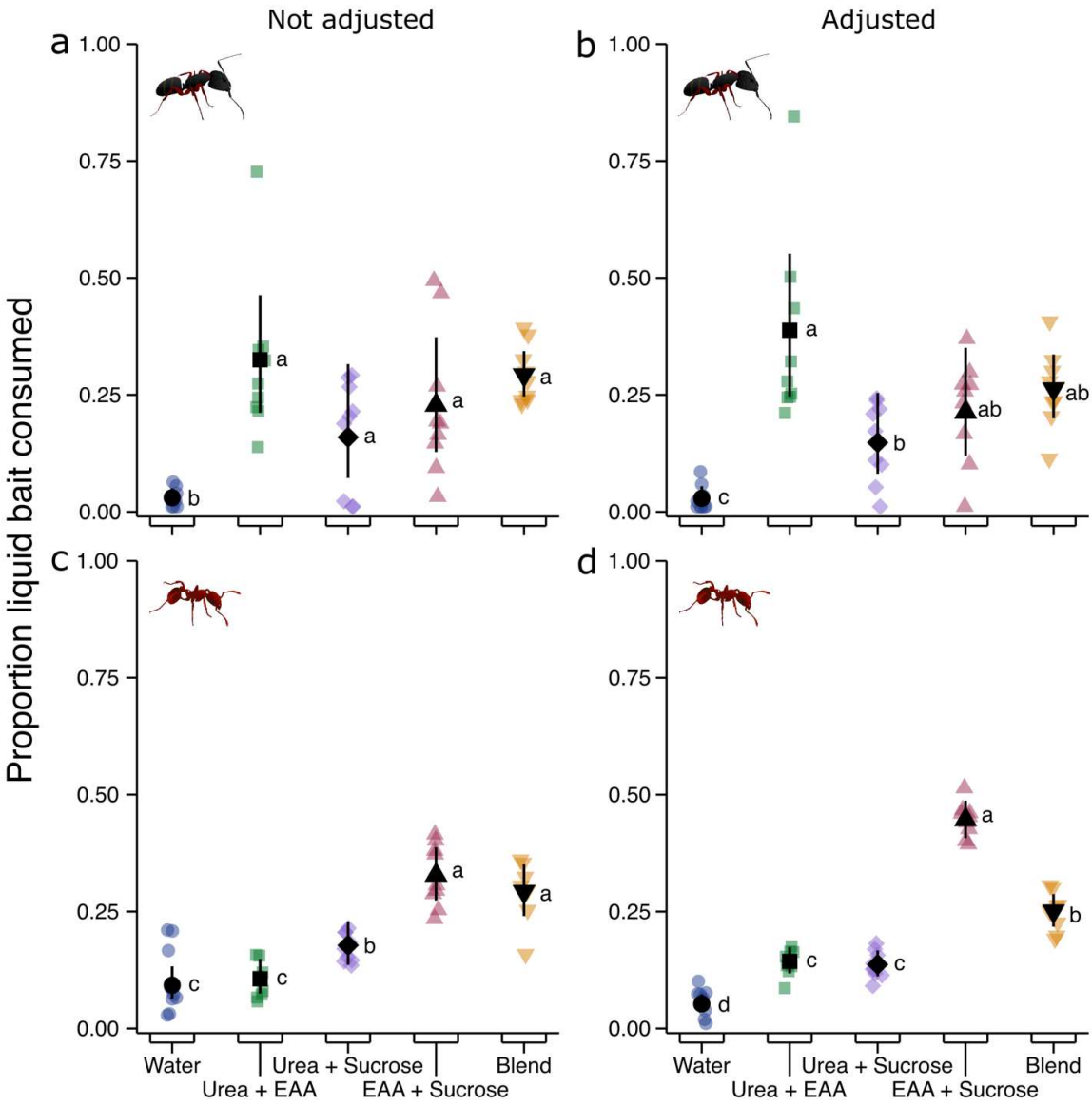

Figure 8.3. **Comparative consumption of liquid food baits by colonies of *Camponotus modoc* carpenter ants [a (n = 9), b (n = 9)] and *Myrmica rubra* fire ants [c (n = 9), d (n = 9)]. Colonies were offered a choice of aqueous solutions of macro-nutrients – urea, essential amino acids (EAAs), and sucrose – that were tested in binary combinations and in a ternary blend ('Blend'). The concentration of binary combinations was either not adjusted [a, c; urea (2.5%) and EAAs (0.55%); urea (2.5%) and sucrose (2.5%); EAAs (0.55%) and sucrose (2.5%)] or adjusted [b, d; urea (4.55%) and EAAs (1.0%); urea (2.775%) and sucrose (2.775%); EAAs (1.0%) and sucrose: 4.55%) to the same total concentration as the ternary blend [urea (2.5%), EAAs (0.55 %), and sucrose (2.5%) w/v)]. Coloured symbols indicate responses of individual colonies (replicates) and black symbols represent modelled estimated marginal means and 95% confidence intervals. Means with different letters are statistically different ($p < 0.05$) in pairwise comparisons (see Table E6).**

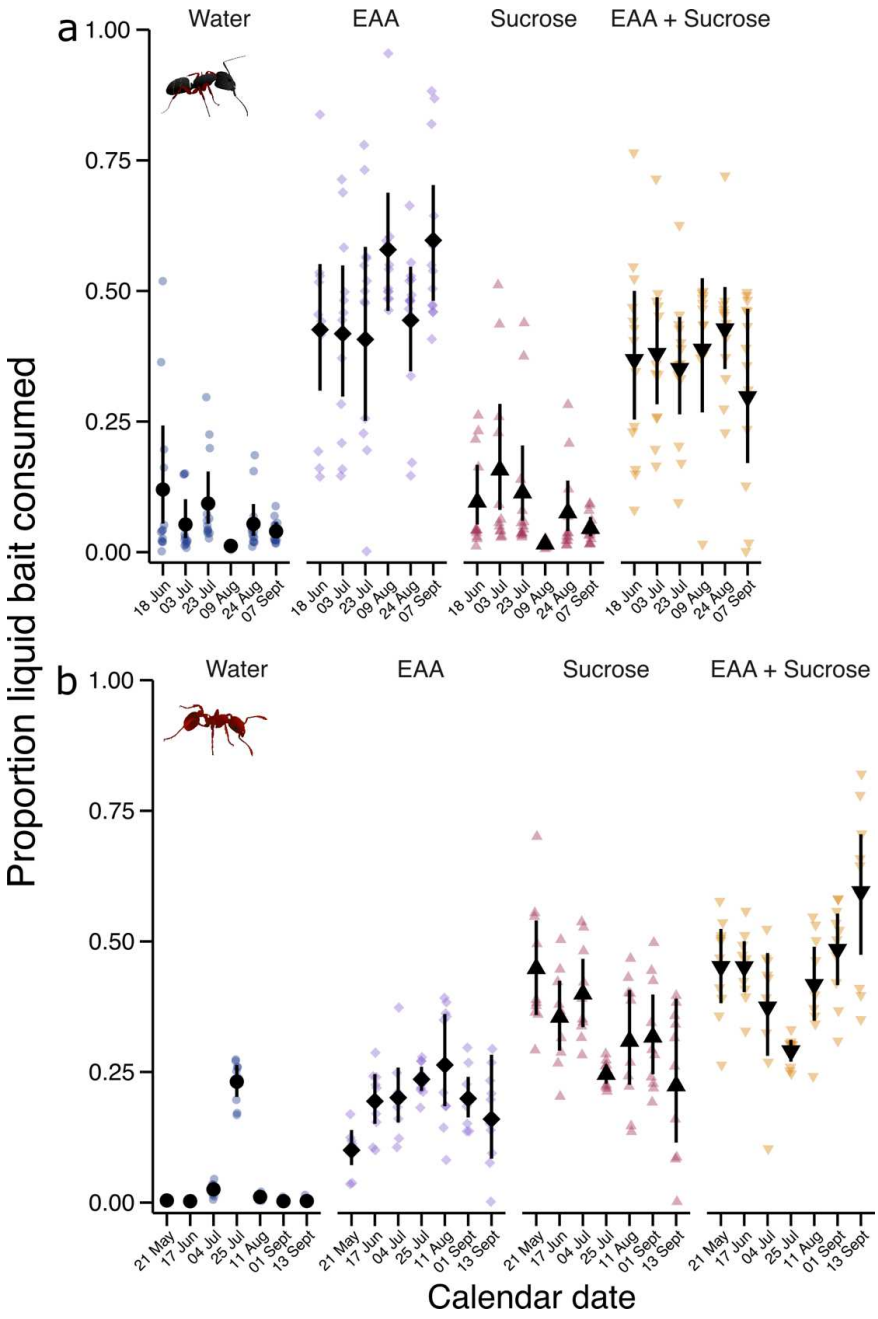

Figure 8.4. **Comparative consumption of liquid food baits by field colonies of *Camponotus modoc* carpenter ants (n = 12–13) (a), and *Myrmica rubra* fire ants (n = 10) (b) during 21 May to 13 September 2021. Colonies were offered a choice between aqueous solutions of essential amino acids [EAAs (5.55%), w/v), sucrose (5.55%, w/v), and both [EAAs (1%), sucrose (4.55%), w/v]. Coloured symbols indicate the responses of individual colonies, and black symbols represent modelled estimated marginal means and 95% confidence intervals. Nutrient solution, date, and interaction between nutrient solution and date, were all significant predictors of bait consumption (see results). Statistical results of pairwise comparisons within date are reported in Table E7.**

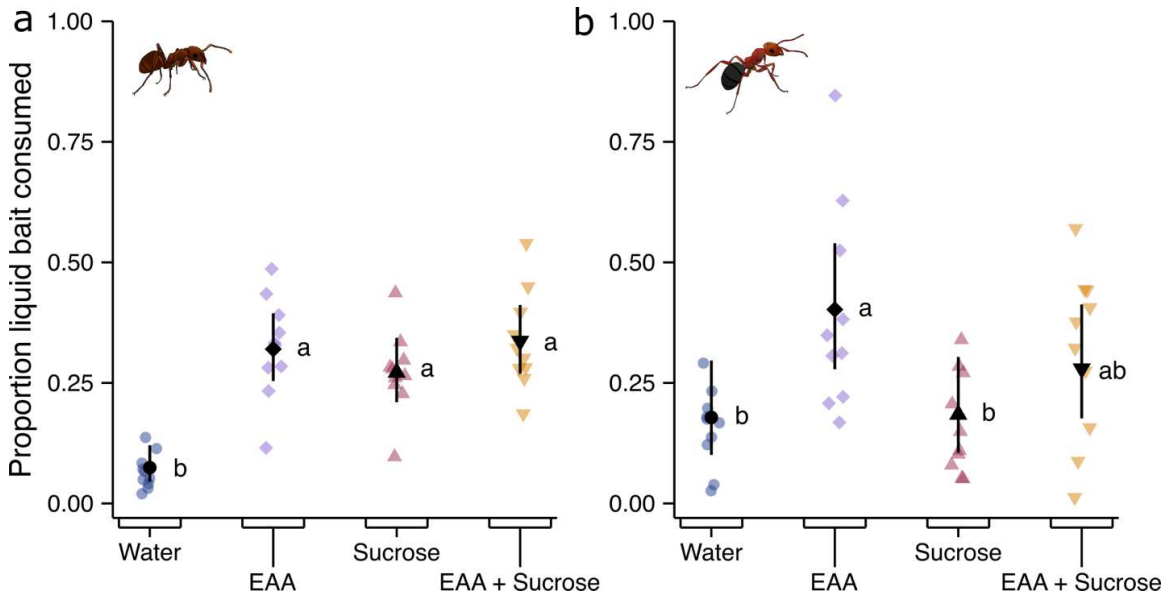

Figure 8.5. **Comparative consumption of liquid food baits by colonies of *Lasius niger* black garden ants (n = 10) and *Formica aserva* thatching ants (n = 10). Colonies were offered a choice between aqueous solutions of essential amino acids [EAAs (5.55%) w/v], sucrose (5.55%, w/v), and both [EAAs (1%); sucrose (4.55%) w/v). Coloured symbols represent responses of individual colonies and black symbols are estimated marginal means and 95% confidence intervals. Treatment (bait composition) was a significant predictor of bait consumption (see results). Means with different letters are statistically different ($p < 0.05$) in pairwise comparisons (see Table E8).**

Chapter 9.

New lethal liquid bait for control of pest ants

A similar version of this chapter has been accepted with minor revisions: Renyard, A., Hoven, K., Pinard, C., and Gries, G., (accepted with minor revisions). New lethal liquid bait for control of pest ants. J. Pest Sci. (Ref: Submission ID dad19e66-593c-4d56-a75b-965462b89438)

9.1. Abstract

An aqueous ant bait consisting of sucrose (4.55% w/v), essential amino acids (1%), and water is known to be highly appealing to multiple ant species throughout the foraging season. Here, we tested whether this bait, combined with boric acid as the lethal agent, has potential for control of pest ants. Our specific research objectives were to: (1) assess bait lethality to diverse species of ants (European fire ants, *Myrmica rubra,* western carpenter ants, *Camponotus modoc*, thatching ants, *Formica obscuripes*); (2) test the effect of boric acid concentration on mortality of *M. rubra* workers and colonies; (3) compare consumption, and demise timeline, of lethal liquid baits and lethal gel baits; and (4) investigate whether lethal liquid baits reduce the size of *M. rubra* colonies. In laboratory experiments (objectives 1–3), the bait induced rapid worker mortality (<22 days) in all three species of ants tested. Increasing the concentration of boric acid from 1% to 5.4% accelerated the demise of only worker ants, but not queen ants, in *M. rubra* colonies, indicating that 1% boric acid is sufficiently lethal. Worker ants of *M. rubra* strongly preferred liquid baits to gel baits of identical nutrient composition, with the former bait accelerating worker demise. In a field experiment (objective 4) in a public park heavily infested with *M. rubra*, the 12 treatment colonies provided with a lethal liquid bait (4.55% sucrose; 1% EAA; 1% boric acid) over 114 days significantly declined, whereas the 12 control colonies provided with the corresponding non-lethal bait did not. The bait, with appropriately adapted bait deployment protocol, should be tested for control of other pest ants, particularly those that preferentially feed on liquid foods.

9.2. Introduction

Pest ants, particularly invasive species, cause significant socioeconomic and environmental damage in various sectors including agriculture, human health, infrastructure, and recreation (Angulo et al. 2022; Gruber et al. 2022). For the period of 1930 to 2020, costs inflicted by invasive ants are estimated at US $10.95 billion in 27 countries (Angulo et al. 2022; Gruber et al. 2022), with predicted costs of $ 40.98 billion for the period of 1980–2084 (Gruber et al. 2022). Invasive pest ants alter the composition and diversity of native animal and plant communities (Lach and Hooper-Bùi 2010), and they disrupt seed dispersal and pollination (Rodriguez-Cabal et al. 2012; Prior et al. 2014; Meadley-Dunphy et al. 2020; Costa et al. 2023). Additionally, invasive ants displace native ants (Sanders et al. 2003), and profoundly impact other insects (Jourdan et al. 2022; Lee and Yang 2022), crustaceans (Lee and Yang 2022), birds (Allen et al. 2004; Jourdan et al. 2022; Lee and Yang 2022), reptiles (Allen et al. 2004; Lee and Yang 2022), amphibians (Allen et al. 2004; Lee and Yang 2022), and mammals (Kamaru et al. 2024).

Controlling pest ants poses significant challenges. Strategies encompassing biological, chemical, and cultural control tactics may be needed for effective pest ant control (Hoffmann et al. 2010, 2016). The advantages and disadvantages of each tactic hinge on factors such as the ant targeted, the environment (e.g., urban, agricultural, natural), and the geographic scale (Hoffmann et al. 2010, 2016). Release of biocontrol agents, such as phorid flies, and dissemination of microsporidia, have been considered for targeted, landscape-level treatment but – to date – biocontrol agents have not been successful in eradicating ant populations (Callcott et al. 2011; Oi et al. 2015; Lee and Yang 2022). Biocontrol agents have weakened, but not annihilated, colonies of red imported fire ants, *Solenopsis invicta* (Callcott et al. 2011; Oi et al. 2015), and biocontrol agents are not widely used for control of other invasive ants (Orr et al. 2001; Heraty et al. 2021; Lee and Yang 2022). Chemical control – spraying broadcast insecticides – is simple and could be considered for spot area treatments, provided the risk to non-target species is minimal. However, excessive use of chemicals in ant control is not desirable due to adverse effects on non-target species (Suiter et al. 2021; Tay 2023), and because queen ants – the reproductive cast of ant colonies – reside within their nests out of reach of insecticides (Gentz 2009). Alternative control tactics, such as trail pheromone

disruption, mass trapping, and dissemination of lethal hydrogel beads, show promise for reducing ant populations but it remains unclear how well they can be integrated into wide-scale pest ant management (Suiter et al. 2021).

Lethal baits offer great prospects for ant control. They rely on the food-sharing (trophallaxis) behaviour by ants, which enables the distribution of lethal agents to nestmate worker and queen ants, and reduces adverse effects on non-target species (Hoffmann et al. 2010). For example, bait deployment is the only tactic that achieved area-wide eradication of invasive ants but only on small scales (<10 hectares) (Hoffmann et al. 2016). To this end, the effectiveness of lethal ant baits hinges upon three criteria: (1) bait attractiveness, (2) bait matrix, and (3) delayed toxicity (Silverman and Brightwell 2008; Hoffmann et al. 2010; Lee and Yang 2022). The bait must be at least as attractive as the ants' preferred food sources to draw the attention of foraging ants. Food sources preferred by ants vary with ant species, ant colony demographics, and the ants' peak foraging season (Blüthgen and Feldhaar 2010; Csata and Dussutour 2019). An ideal bait remains attractive through the entire foraging season. Adult ants ingest primarily liquid foods due to the narrow constriction at their petiole, but they collect solid food such as prey for larval offspring (Dussutour and Simpson 2009; Richter and Economo 2023). The propensity of adult forager ants to pick up solid bait varies by species. To date, most baits deployed in eradication trials were solid baits of corn grit (Hoffmann et al. 2016). Whereas myrmicine ants (e.g., *S. invicta*) readily forage on corn grit baits, formicine ants (e.g., yellow crazy ants, *Anoplepsis gracilipes*) and dolichoderine ants (e.g., Argentine ants, *Linepithema humile*) preferentially forage on liquid baits (Rust and Su 2012). Delayed toxicity of baits ensures that forager ants can carry the lethal bait to their nest and distribute it among their nestmates and queen(s) (Stringer et al. 1963; Rust et al. 2004). Ant baits with sustained attractiveness to many species, particularly liquid feeders, would greatly improve integrated ant control (Hoffmann et al. 2010).

Recently, we have developed a blend of macro-nutrients that is appealing to multiple ant species throughout the foraging season (AR et al., unpubl. data). The blend consists of sucrose – a preferred sugar of ants (Cornelius et al. 1996; Blüthgen and Fiedler 2004; Zhou et al. 2015; Renyard et al. 2021) – and essential amino acids (EAAs) (Straka and Feldhaar, 2007; AR et al., unpubl. data) in an aqueous solution (hereafter 'Sucrose/EAA bait'). This bait was readily consumed by most species of ants tested to

date (carpenter ants, *Camponotus modoc;* European fire ants, *Myrmica rubra;* black garden ants, *Lasius niger;* thatching ants, *Formica oreas*; brood raiding ants, *Formica aserva*; AR et al., unpubl. data). Moreover, in a field trial with *C. modoc* and *M. rubra,* the Sucrose/EAA bait was consumed as much as the EAA-only bait (*C. modoc*), or the sucrose-only bait (*M. rubra*), throughout the entire ant foraging season (AR et al., unpubl. data), suggesting that both types of macro-nutrients could be combined in the same bait for attraction of both 'sugar- and protein-loving ants'.

Here, we investigated the efficacy of our Sucrose/EAA bait, containing boric acid as the lethal agent, for ant control. We selected boric acid as the lethal agent because of its low toxicity to non-target species (United States Environmental Protection Agency 1993; Harper et al. 2012) and slow killing speed (Klotz and Moss 1996; Rust et al. 2004). We tested the effect of both boric acid concentration and bait matrix (liquid, gel) because both parameters affect bait performance (Hooper-Bui and Rust 2000; Silverman and Roulston 2001).

We worked with *M. rubra*, *C. modoc,* and *F. oreas* as model species. *Myrmica rubra* is a temperate ant native to Eurasia but has become invasive in temperate regions of Eastern and Western Canada and the USA (Wetterer and Radchenko 2010). *Myrmica rubra* is an aggressive soil-dwelling ant with a painful sting, altering the composition of arthropod and plant communities in its invaded range (Naumann and Higgins, 2015; Meadley-Dunphy et al., 2020). *Camponotus modoc* inhabits coniferous forests of Western North America (Hansen and Akre 1985) but also nests in human-made wooden structures (Hansen and Klotz 2005). *Formica oreas* occurs in Western Canada and the USA and was included in our study for taxonomic diversity.

Our objectives were to: (1) assess bait lethality to diverse species of ants (*M. rubra, C. modoc,* and *F. oreas*); (2) test the effect of boric acid concentration on mortality of *M. rubra* workers and colonies; (3) compare consumption, and demise timeline, of lethal liquid baits and lethal gel baits; and (4) investigate whether lethal liquid baits reduce the size of *M. rubra* field colonies.

9.3. Material and Methods

9.3.1. Collection and maintenance of ants

Myrmica rubra was collected and reared as previously detailed (Hoefele et al. 2021; Renyard et al. 2021), with some modifications. In the summer of 2022, we collected 12 colonies at Inter River Park (North Vancouver, BC, CA) for laboratory feeding trials with ants. Colonies were dug up with their nesting soil, temporarily placed in glass jars (1L), and then transferred to 12 glass containers (26 × 21 × 40.6 cm) maintained indoors under a natural daylength cycle and at 22 °C. To enable air exchange and prevent ant escape, container lids were fitted with mesh holes and upper inner container walls were coated with an equal mix of Vaseline (Unilever, London, UK) and mineral oil (Anachemia, Lachine, QC H8R1A3, CA). The soil surface served as the ants' foraging area which was provisioned with apples, deceased cockroaches, and 20% sugar water *ad libitum*. Every two weeks, water was added to the soil to ensure adequate moisture content.

To collect *F. oreas* colonies, thatch mounds and soil were dug up along roadside ditches near Port Kells (Surrey, BC, CA) and placed into large plastic bins (67.3 × 42.9 × 34.5 cm) for transport to the Science Research Annex (49°16′33″ N, 122°54′55″ W) on the Burnaby campus of Simon Fraser University (SFU). There, these large bins were connected to smaller plastic bins (37.6 × 24.4 ×23.6 cm) via polyvinylchloride (Nalgene™) tubing and barbed plastic plumbing fixtures, thus allowing ants to move between bins. The smaller bins served as the ants' foraging area and were provisioned with food and sugar water (see above). Air exchange and retention of ants were achieved as described for *M. rubra.*

Colonies of *C. modoc* were collected in coniferous forests and maintained as previously described (Renyard et al. 2019), with slight modification. Briefly, infested log sections were excised and transferred to large plastic bins (64 × 79 × 117 cm) which were kept in an outdoor, under-cover area of the Science Research Annex. The bins were connected to glass containers (30.5 × 26 × 50.8 cm) which served as the ants' foraging area and were provisioned with food and sugar water (see above). Air exchange in aquaria and retention of ants were achieved as described for *M. rubra*.

9.3.2. Preparation of liquid and gel baits

To prepare liquid (aqueous) lethal baits, sucrose (4.55%), 11 essential amino acids (EAAs, 1%; Table 9.1) and boric acid (1, 2, 3 or 5.4 %) were dissolved under stirring in distilled water (50% of desired volume), after which more distilled water was added to reach the target weight by volume (w/v) solution. Liquid non-lethal baits were prepared similarly except that no boric acid was added. For efficiency, baits were prepared in large batches, and 1- and 8-mL bait aliquots were pipetted into 1.5-mL Eppendorf tubes and 15-mL Falcon tubes, respectively, which were then frozen until needed. Prior to deployment of bait tubes, they were thawed, and a 1-cm^3 piece of cotton dental wick (Richmond Dental & Medical, Charlotte, NC 28205, USA) and a cotton ball were stuffed into Eppendorf and Falcon tubes, respectively, to retain the liquid bait while still enabling bait consumption by ants without spillage.

To prepare lethal gel baits, powdered gelatin (1.42%; Knox brand, TreeHouse Foods, Inc; Il, USA), sucrose (4.5%), essential amino acids (1%), and boric acid (1%) were thoroughly mixed in a beaker, after which cold distilled water (10 mL) was added under stirring. When the mixture was semifluid, boiling water (10 mL) was added under stirring until all solutes were fully dissolved. Then, the mixture was transferred to a graduated cylinder, topped up with water for a total volume of 48 mL, poured back into the beaker and thoroughly mixed. Aliquots (1 mL) of this mixture were pipetted into 1.5-mL Eppendorf tubes which were refrigerated, uncapped, for 1 h to solidify the mixture. Unlike liquid baits, gel baits could not be frozen for preservation, and were prepared, and kept refrigerated, in batches sufficient to feed laboratory ant colonies for 2 weeks. Non-lethal gel baits were prepared following the same protocol except that no boric acid was added.

9.3.3. General laboratory bioassay procedure

To assess bait appeal and lethality in various laboratory experiments (Objectives 1–3), we tested *M. rubra* colonies, 12-worker groups of *C. modoc*, and 20-worker groups of *F. oreas*. Colonies of *M. rubra* consisted of two queens and 100 workers sorted from field-collected ants. Colonies were housed in escape-proof (see above) Tupperware

containers (17 × 17 × 6 cm) with a mesh-covered hole in the lid for air exchange. Containers were fitted with a 10-mL test tube filled halfway with water and plugged with a cotton ball to provide a humid environment. Groups of *C. modoc* workers (including workers of various sizes) were sorted from six laboratory colonies, and were housed in Tupperware containers (24.4 × 12.7 × 8.9 cm) fitted with a 20-mL test tube as a water reservoir (see above). Groups of *F. oreas* workers (including workers of various sizes) were sorted from four laboratory colonies, and were housed in Tupperware containers (17 × 17 × 6 cm) fitted with a 20-mL test tube as a water reservoir. All containers were kept at 22 °C and a photoperiod of 12L:12D. Prior to the onset of any experiment, any dead workers were replaced with live ones. Every two days, ant colonies or groups of ants were provisioned with one or two bait-containing Eppendorf tubes, and deceased ants were counted.

9.3.4. Specific experiments

Objective 1: Assess bait lethality to diverse species of ants (lab experiments)

To concurrently assess both bait lethality and potential bait aversion (Exps. 1–3), treatment colonies and treatment groups of *M. rubra* (n = 8), *C. modoc* (n = 6), and *F. oreas* (n = 8) – each containing all castes and worker ants of all sizes – were offered a choice between a lethal and a non-lethal bait, each bait containing 4.55% sucrose and 1% EAAs but only the lethal bait containing 1% boric acid (Fig. 9.1a). Conversely, corresponding control colonies and control groups of *M. rubra*, *C. modoc,* and *F. oreas* were offered two non-lethal baits. Deceased ants were counted 24 h after experiment initiation and then every 48 h until all ants were deceased.

Objective 2: Test the effect of boric acid concentration on mortality of M. rubra workers and colonies (lab experiments)

The effect of boric acid concentration on the mortality of *M. rubra* workers and colonies was tested in experiments 4 and 5. In experiment 4, we prepared four liquid test baits, each containing 4.55% sucrose and 1% EAAs but variable amounts of boric acid. Groups of five worker ants from each of four laboratory colonies were collected and placed in separate Tupperware containers (17 × 17 × 6 cm) that contained a single 50-μL droplet of a liquid test bait containing boric acid at 0, 1, 2 or 3%. Once an ant had fed

on the droplet – as evidenced by moving mouthparts indicative of bait ingestion – the ant was transferred to an amber-glass test tube (10 mL) filled halfway with a water reservoir which was secured with a cotton ball. After placing the ant in the test tube, its opening was closed with another cotton ball. Any ants that did not feed were replaced with ants from the same colony. Procedures were repeated until each of the four baits had been fed on by 20 ants. All test tubes were checked for ant mortality after 1, 2, 4, 8, 12, and 24 h, then every 12 h for 5 days, and finally every 24 h for 21 days.

In experiment 5, we tested the effect of boric acid concentration (1% or 5.4%, the latter concentration commonly found in commercial baits) on the mortality of *M. rubra* colonies. Liquid baits were prepared with the same nutrient content (4.55% sucrose, 1% EAA) but a dissimilar boric acid concentration (1% or 5.4%). As boric acid did not cause bait aversion by ants in experiment 1–3 (see Results), there was no need to offer colonies a choice between lethal and non-lethal baits. Consequently, six colonies each were offered a single bait with 1% boric acid or a single bait with 5.4% boric acid. Every two days, bait tubes were replaced, and deceased worker and queen ants were counted, until all ants in all colonies were deceased.

Objective 3: Compare consumption, and demise timeline, of lethal liquid and gel baits (lab experiments)

In Experiment 6, 12 laboratory colonies of *M. rubra* were offered a choice between a liquid bait and a gel bait, testing for preferential bait consumption (Renyard et al., 2021). One day prior to the experiment, all colonies were food-deprived, and gel baits (see above) were prepared and kept refrigerated. On the day of the experiment, frozen liquid baits (see above) in 1.5-mL Eppendorf tubes were thawed, stuffed with a cotton wick to retain the liquid, and both liquid and gel baits were weighed to the nearest 0.0001 g (TR 204 scale; Denver Instrument Company, CO, USA). For each replicate, liquid and gel bait Eppendorf tubes were taped to the edge of jar lids (diam: 15cm), with their position on the lid randomly selected. To determine the weight loss of baits that was due to evaporation, rather than consumption by ants, one Eppendorf tube with liquid bait and one with gel bait were taped, inaccessible to ants, to the underside of bioassay container lids. To initiate replicates, all Eppendorf tubes were uncapped, and jar lids carrying the two bait tubes were placed on the soil surface inside containers. After ants had foraged for 6 h, all tubes were capped and reweighed. Bait consumption was

determined as the weight differential of Eppendorf tubes fed on by ants *minus* the weight differential of corresponding evaporation control tubes.

In experiment 7, we compared the lethality of liquid and gel baits to *M. rubra*, both baits containing 1% boric acid. Six colonies each received either a liquid or a gel bait. Every two days, baits were replaced and deceased ants were counted, until all ants in all colonies were deceased.

Objective 4: Investigate whether liquid baits reduce the size of M. rubra field colonies (field experiment)

Experiment 8 tested the ability of the lethal liquid bait (4.55% sucrose; 1% EAA; 1% boric acid) to reduce the size of *M. rubra* colonies in a field setting. Two ant-infested plots, which were separated by a 10-m natural land constriction, were selected at Inter River Park (North Vancouver, BC, Canada). In each plot, 12 colonies were flagged (Fig. 9.1b,c) which were at least 2 m apart. Treatment- and control-plot colonies were baited with the lethal bait (4.55% sucrose; 1% EAA; 1% boric acid) and the non-lethal liquid bait (4.55% sucrose; 1% EAA), respectively, which were replaced every day from Monday to Friday for 16 weeks. Twice per week (consistently between 08:30–11:30), colony demise was monitored by placing apple baits in petri dish lids (40 mm diam) next to colonies, photographing ants on apple baits 70 min later, and eventually counting ants on photographs using the cell counter tool in FIJI (V2.9.0/1.53t). Apple baits were prepared from 0.5-cm thick slices of ambrosia apples that were punched out into 19-mm discs with a cork cutter. Bait tubes and ant-monitoring apple baits were covered with a Unitrap lid (16.2 cm diameter; Forestry Distributing, Boulder, CO, USA) (Fig. 9.1c) to provide weather protection and prevent bait tampering by animals.

During days 1–74 of the experiment, lethal and non-lethal liquid baits were presented in 1.5-mL Eppendorf tubes. When we noticed, around day 74, that both lethal and non-lethal bait reservoirs were empty at the time baits were replaced, 15-mL Falcon tubes (which held 8 mL of liquid bait) instead of 1.5-mL Eppendorf tubes were deployed to ensure sustained bait availability for ants.

9.3.5. Statistical analyses

Data were analysed in R (v. 4.2.2) and R studio (v. 2022.07.1+554) (R Core Team 2022), using generalized linear mixed models (GLMM; Bolker et al. 2009; Winter 2019). The response variable was the proportion of surviving ants as a function of treatment (lethal bait *vs.* non-lethal bait), days elapsed since the experiment started, and the interaction between treatment and experiment day, with each ant colony, or group of ants, as a random intercept. Data of experiments 1–3 and 5 were analyzed using a binomial distribution and a logit link function, and data of experiment 7 were analyzed using a beta-binomial distribution and a logit link function. For data in experiment 4, we ran a survival analysis, and compared survival probability using a log-rank test.

For data analyses of experiment 6, we calculated bait consumption by ants as the weight differential of Eppendorf tubes before and after ant feeding *minus* the weight differential of corresponding evaporation control tubes (see above). In some ant colonies that consumed little (if any) of a particular bait type, bait consumption data became less than 0 following weight loss subtraction due to passive water evaporation measured in evaporation controls. As bait consumption data could not be less than 0, we assigned 0-values to these data. Subsequently, we calculated the proportion of bait consumed by each ant colony by dividing the consumption of each type of bait by the total bait consumption. We then applied a standard transformation to restrict bait consumption values between 0 and 1 (Smithson and Verkuilen 2006), as we fit a GLMM with a beta distribution and a logit link function. Transformed proportional bait consumption was the response variable, with type of bait as predictor variable, and ant colony as a random intercept.

For data analyses of field experiment 8, we initially fitted the number of ants counted on apple monitoring baits as a function of treatment (lethal bait *vs.* non-lethal bait), experiment day, interaction between treatment and day, mean daily temperature, and days after switching Eppendorf bait tubes to Falcon bait tubes (which have a larger bait reservoir; see above). The latter two variables were removed due to multicollinearity with other variables. Therefore, the final model, a tweedie distributed GLMM with a log link function, fitted the number of ants on apple monitoring baits as a function of treatment (lethal bait *vs.* non-lethal bait), experiment day, and interaction between

treatment and day, with ant colony as a nested random effect within the treatment plot and the control plot.

For each statistical model, we verified model fit, and inspected residual normality, variance homogeneity, and over/under dispersion patterns, using the DHARMa package (Hartig 2022). To improve model fit for experiments 1–3, 5 and 7, we removed day 0 (the day the experiment started), because – by definition – all proportions would equal 1. All models were fitted using the glmmTMB package (Brooks et al. 2019), and data were processed using the tidyverse functions (Wickham et al. 2019), and the plyr package (Wickham 2011). Following assessments of model fit, we assessed the significance of various predictors in our models using a likelihood ratio test. For experiments 4 and 8, we obtained back-transformed means and confidence intervals, using the emmeans package (Lenth 2023). We produced graphics in R studio, using ggplot2 (Wickham 2016) and ggprism (Dawson 2022), and ran final assembly and editing of figures in Inkscape (v. 1.0.2). Code and analyses were uploaded to a repository and can be accessed at (Renyard et al. 2024).

9.4. Results

Objective 1: Assess bait lethality to diverse species of ants

Testing *M. rubra* colonies (Exp. 1), at day 9 (midway through the experiment), proportionally (mean proportion; 95% confidence interval) fewer workers were still alive in treatment colonies feeding on the lethal bait (0.03; 0.02–0.06) than in control colonies feeding on the non-lethal bait (0.85; 0.78–0.91) (Fig. 9.2a). There was a significant effect of treatment (lethal *vs.* non-lethal bait) (χ^2 = 1739.2, d. f. = 2, p < 0.0001), day in experiment (χ^2 = 3836.1, d. f. = 2, p < 0.0001), and interaction between treatment and day (χ^2 = 1696.6, d. f. = 1, p < 0.0001) on worker ant survival. Similarly, proportionally fewer queens were still alive in treatment colonies (0.05; 0.01–0.26) than in control colonies (0.99; 0.89–1.0) (Fig. 9.2b). Again, there was a significant effect of treatment (χ^2 = 2524.6, d. f. = 2, p < 0.0001), day in experiment (χ^2 = 4621.5, d. f. = 2, p < 0.0001), and interaction between treatment and day (χ^2 = 2481.9, d. f. = 1, p < 0.0001; Fig. 9.2b) on queen ant survival. In treatment colonies, all workers and queens were deceased on days 18 and 15, respectively.

Testing groups of *F. oreas* workers (Exp. 2), at day 11, proportionally fewer workers were still alive in treatment groups (0.09; 0.04–0.17) than in control groups (0.74; 0.58-0.86) (Fig. 9.2c). There was a significant effect of treatment (χ^2 = 338.74, d. f. = 2, p < 0.0001), day in experiment (χ^2 = 1592.7, d. f. = 2, p < 0.0001), and interaction between treatment and day (χ^2 = 321.12, d. f. = 1, p < 0.0001). All workers in treatment groups were deceased by day 22.

Testing groups of *C. modoc* workers (Exp. 4), by day 7, proportionally fewer workers were still alive in treatment groups (0.16; 0.08–0.29) than in control groups (0.84; 0.73–0.91) (Fig. 9.2d). There was a significant effect of treatment (χ^2 = 104.87, d. f. = 2, p < 0.0001), day in experiment (χ^2 = 697.56, d. f. = 2, p < 0.0001), and interaction between treatment and day (χ^2 = 79.379, d. f. = 1, p < 0.0001). All workers in treatment groups were deceased on day 14.

Objective 2: Test the effect of boric acid concentration on mortality of M. rubra workers and colonies

A single feeding bout by *M. rubra* workers on baits that contained boric acid at 0, 1, 2, or 3% (Exp. 5) did not differentially affect their survival time (log-rank test: χ^2 = 0.4, d. f. = 3, p = 0.9; Fig. 9.3a), indicating that boric acid does not induce concentration-dependent mortality after a single feeding bout, at least not at the concentrations (1–3%) tested in this experiment.

When we tested the effect of boric acid concentration (1% or 5.4%) on the survival time of 100 workers and two queens in each of 12 *M. rubra* colonies (Exp. 6), at day 20, proportionally fewer worker ants were still alive in colonies feeding on the 5.4% boric acid bait (0.03; 0.02–0.07) than in colonies feeding on the 1% boric acid bait (0.15; 0.08–0.26) (Fig. 9.3b). There was a significant effect of treatment (1% or 5.4% boric acid; χ^2 = 12.61, d. f. = 2, p = 0.002), day in experiment (χ^2 = 12592, d. f. = 2, p < 0.0001), and interaction between treatment and day (χ^2 = 6.9141, d. f. = 1, p = 0.009; Fig. 9.3b) on worker ant survival. Worker ants in all colonies were deceased by day 38. Conversely, queen ant survival was not differentially affected by boric acid concentration (χ^2 = 1.32, d. f. = 2, p = 0.52; Fig. 9.3c). At day 20, proportionally as many queens survived ingestion of boric acid at 5.4% per bait (0.54; 0.23–0.82) and 1% per bait (0.51; 0.21–0.80), with the high and low boric acid concentration killing all queens by days 30

and 34, respectively. There was a significant effect of day in experiment on queen ant survival (χ^2 = 320.84, d. f. = 2, $p < 0.0001$; Fig. 9.3c) but no significant effect of treatment and day interaction (χ^2 = 1.31, d. f. = 1, $p = 0.25$).

Objective 3: Compare consumption, and demise timeline, of liquid and gel baits

When we offered *M. rubra* colonies a choice between a non-lethal liquid bait and a non-lethal gel bait (Exp. 6), colonies consumed greater proportions of liquid baits (0.93; 0.89–0.95) than of gel baits (χ^2 = 63.67, d. f. = 1, $p < 0.0001$; Fig. 9.4a).

By day 36 (the midpoint of the experiment), proportionally fewer worker ants were still alive in colonies feeding on lethal liquid baits (0.005; 0.003–0.007) than in colonies feeding on lethal gel baits (0.05; 0.03–0.07). Liquid and gel baits killed all ants by day 39 and 73, respectively. There was a significant effect of treatment (liquid bait *vs.* gel bait; χ^2 = 286.55, d. f. = 2, $p < 0.0001$), day in experiment (χ^2 = 1625.3, d. f. = 2, $p < 0.0001$) and interaction between treatment and day (χ^2 = 270.95, d. f. = 1, $p < 0.0001$) (Fig. 9.4).

Objective 4: Investigate whether lethal liquid baits reduce the size of M. rubra field colonies

The lethal liquid bait reduced the size of *M. rubra* colonies in Inter River Park (Exp. 8) and thereby the overall *M. rubra* infestation (Fig. 9.5). There was a significant effect of treatment (lethal bait *vs.* non-lethal bait) (χ^2 = 487.92, d. f. = 66, $p < 0.0001$), day in experiment (χ^2 = 1378.7, d. f. = 128, $p < 0.0001$), and interaction between treatment and day (χ^2 = 414.09, d. f. = 64, $p < 0.0001$). Initially, fewer ants were counted on apple monitoring baits in the lethal treatment plot than in the non-lethal control plot but the difference was not statistically significant ($p > 0.05$; Table 9.2; Fig. 9.5). After 33 days, numbers of ants on apple baits spiked in both the treatment and the control plot and remained similarly high until day 71 ($p > 0.05$; Table 9.2; Fig. 9.5). Following day 71, numbers of ants on apple baits sharply declined in the treatment plot but not the control plot ($p < 0.05$; Table 9.2; Fig. 9.5).

9.5. Discussion

Deployment of our lethal liquid bait shows great promise as a new tactic for controlling pest ants. In the laboratory, the bait proved lethal to diverse ant taxa, including *M. rubra* workers and queens, *C. modoc* workers, and *F. oreas* workers. Increasing the concentration of boric acid (as the lethal agent in the bait) from 1% to 5.4% slightly accelerated the demise of worker ants, but not queen ants, in *M. rubra* colonies (Fig. 9.3), indicating that a 1% boric acid is sufficient. Workers of *M. rubra* preferred liquid baits to gel baits, with the former bait accelerating worker demise. In the field, *M. rubra* colonies feeding on lethal liquid baits declined, resulting in a lower *M. rubra* infestation (Fig. 9.5). Whereas known liquid baits for ants typically contain sucrose as the macro-nutrient and feeding stimulant (Klotz et al. 1996; Silverman and Roulston 2001; Daane et al. 2006; Lee and Yang 2022; McCalla et al. 2023), our liquid bait contains both sucrose and essential amino acids, thus making the bait appealing to both 'sugar- and protein-loving ants' (AR et al., unpubl. data). Moreover, even if pest ants were to shift their macro-nutrient preference from carbohydrates to proteins, or *vice versa*, over the course of the foraging season, the bait retains its season-long appeal to foraging ants (AR et al., unpubl. data) because both macro-nutrients are bait constituents.

The performance of ant baits depends on bait properties. Effective baits are formulated with attractive and palatable food components, are not repellent, and cause delayed toxicity (Stringer et al. 1963; Rust et al. 2004; Hoffmann et al. 2010). Our bait meets and exceeds these criteria, as follows: (1) with a nutrient blend composition of sucrose and essential amino acids, the bait is 'appealing' to ants in diverse taxa, and more appealing to some taxa than a sucrose-only bait (AR et al., unpubl. data); (2) the bait's appeal spans the entire seasonal activity periods of field-tested ants (AR et al., unpubl. data); (4) boric acid – as the lethal agent in the bait – does not cause bait aversion by ants (this study; Fig. 9.2); (5) the bait's toxicity induces delayed, but significant, ant mortality (this study; Fig. 9.2); and (6) a liquid formulation of the bait is superior to a gel formulation in terms of bait consumption by ants and bait lethality (this study; Fig. 9.4a,b). Generally, liquid baits are highly appealing to ant taxa (Silverman and Roulston 2001; Lee 2008), and are more lethal than gel baits to *L. humile* (Silverman and Roulston 2001). Adult ants are adapted to uptake liquid food which is

easier to imbibe and to share than solid food which must first be digested by ant larvae before it can be shared (Hölldobler and Wilson 1990).

Insecticide type is a significant determinant of successful ant eradication (Hoffmann et al. 2016). We opted for boric acid as the lethal agent in our bait because it has comparatively low toxicity to non-target organisms (United States Environmental Protection Agency 1993; Harper et al. 2012). Nonetheless, boric acid caused 100% mortality of all ants tested (*M. rubra, C. modoc, F. oreas*) but single feeding bouts by individual ants on baits with or without boric acid did not cause differential mortality (Fig. 9.3a), and prolonged consumption of lethal baits was required to induce colony mortality (Fig. 9.3b,c). All data combined indicate that forager ants in field settings would be able to spread the lethal bait among nestmates and larval offspring, as is needed for the demise of colonies. Our findings favorably compare with those in previous studies showing that continuous exposure to lethal baits was necessary to achieve 100% mortality of Argentine ants, ghost ants, *Tapinoma melanocephalum,* and pharaoh ants, *Monomorium pharaonis* (Klotz et al. 1996; Hooper-Bui and Rust 2000). As a higher concentration of boric acid (5.4% *vs.* 1%) did not markedly expedite mortality of *M. rubra* colonies in a laboratory experiment (Fig. 9.3b,c), we field tested baits with only 1% boric acid to keep adverse impact on non-target organisms to a minimum.

The time required to reduce pest ant populations in the field is linked to bait properties (see above), bait deployment protocol (Nelson and Daane 2007), and ant population size (Daane et al. 2006), as reflected in our study with *M. rubra* at Inter River Park (Fig. 9.5). Counts of *M. rubra* worker ants on apple monitoring baits indicated equally large colonies between days 40 to 70 in both the treatment plot (treated with lethal boric acid baits) and the control plot (treated with non-lethal baits), but also revealed that treatment-plot colonies – unlike control-plot colonies – declined 81% between days 40 and 114 (Fig. 9.5). As quantitatively sufficient bait is key for pest ant management (discussed in Daane et al., 2006), the insufficient bait volume (1 mL) during the first part of the study probably caused the delay in treatment-colony decline. Ever since we increased the bait volume from 1 mL (in Eppendorf tubes) to 8 mL (in Falcon tubes), treatment-colonies steadily declined (Fig. 9.5). Statistically, however, we could not link the timing of bait volume change to ant colony decline due to collinearity of this change with other variables in our statistical model. Deployment of large-volume baits from the onset of our study might have achieved faster, and possibly complete,

demise of treatment-plot colonies. This prediction is supported by reports that liquid boric acid baits achieved 100% control of a yellow crazy ant population, *Anoplolepis gracilipes,* in 56 days (Chong and Lee 2009).

As part of the bait deployment protocol, we replaced all baits on five consecutive days per week for 16 weeks. This work-intensive protocol was necessary to prevent spoilage of non-lethal baits but was not necessary for lethal baits, and would not be practical for operational ant control. Boric acid in lethal baits has anti-microbial properties (Güzel et al. 2016; Hernandez-Patlan et al. 2019) that prevent bait spoilage, thus allowing replacements of baits not before the entire bait volume has been consumed after extended bait deployment in the field.

The successful deployment of lethal ant baits for ant control requires careful consideration of the ant species, context (e.g., urban, agricultural, or natural setting), scale, cost, and environmental impact. Invasive ants that do not engage in nuptial flights but, instead, spread by 'budding' (one or more fertile queens and a group of workers leaving an established nest and moving to a new nest site) are more amenable to eradication because infestations have well defined boundaries and expand relatively slowly (Silverman and Brightwell 2008). In its invaded range, *M. rubra* spreads by budding, and thus becomes a good target for eradication by baits. The liquid formulation of a future commercial bait will have greater appeal to *M. rubra* than a gel-type formulation because *M. rubra* colonies preferred liquid baits to gel baits when they were offered a choice (this study, Fig. 9.4a). Liquid baits will be most effective for control of any ants that readily feed on liquid food sources, such as Argentine ants (Silverman and Brightwell 2008) and yellow crazy ants (Lee and Yang 2022). Liquid baits can achieve ant control in both urban (Klotz et al. 1998, 2009; Chong and Lee 2009) and agricultural settings (Klotz et al. 2003; Daane et al. 2006; Greenberg et al. 2006; McCalla et al. 2023), but deploying liquid baits on a large scale is challenging. Both setting up and maintaining bait stations are more labour-intensive (Daane et al. 2006) and costly than sprays. Hydrogel beads can easily be scattered but rapidly lose their attractiveness due to dehydration (Buczkowski et al. 2014; Tay et al. 2017; McCalla et al. 2020). Solid baits remain effective for extended periods of time and can be scattered over large areas but they are less appealing to liquid-feeding ants (Baker et al., 1985; Lee, 2008; Boland et al., 2011; Nyamukondiwa and Addison, 2014).

In conclusion, our bait's composition of sucrose, essential amino acids, and boric acid as the lethal agent caused rapid mortality in all ant species tested in this study (*M. rubra*, *C. modoc*, *F. oreas*). The bait significantly reduced the size of *M. rubra* colonies at Inter River Park, and should be tested, with modified bait deployment, for control of other pest ants, particularly those that preferentially feed on liquid foods. The considerable deployment costs of bait stations in operational ant control are outweighed by the substantial economic and environmental costs inflicted by pest ants (Angulo et al. 2022). Moreover, bait stations introduce less insecticide into the environment than sprays (Drees et al. 2013; McCalla et al. 2020), and have lower impact on non-target organisms (Buczkowski, 2020; Hoffmann and Quinn, 2022; but see Hoffmann et al., 2023).

9.6. Data Availability

Data are available from Mendeley Data and can be accessed at: DOI: 10.17632/ry6t7j76wv.1

9.7. Acknowledgements

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9.8. References

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9.9. Tables

Table 9.1. Research objectives (O), ant species (*Myrmica rubra, Formica oreas, Camponotus modoc*) bioassayed, stimuli tested, and numbers of replicates (n) run in experiments (Exp.) 1–8.

Exp. #	Stimuli tested	Species tested (n)
(O1) Assess bait lethality to diverse species of ants		
1–3	Liquid lethal bait[a] (1%[b] boric acid) *vs.* liquid non-lethal bait	*M. rubra* (8); *C. modoc* (*6*); *F. oreas* (8)
(O2) Test the lethality of boric acid concentration on ant workers (Exp. 4) and colonies (Exp. 5)		
4	Liquid lethal bait (1, 2, or 3 % boric acid) *vs.* liquid non-lethal bait	*M. rubra* (20)
5	Liquid lethal bait (1% boric acid) *vs.* liquid lethal bait (5.4% boric acid)	*M. rubra* (6)
(O3) Compare consumption (Exp. 6), and demise timeline (Exp. 7), of lethal liquid and lethal gel baits		
6	Non-lethal liquid bait *vs.* non-lethal gel bait	*M. rubra* (12)
7	Liquid lethal bait (1% boric acid; water) *vs.* gel lethal bait (1% boric acid)	*M. rubra* (12)
(O4) Investigate whether liquid lethal baits reduce the size of M. rubra field colonies		
8	Liquid lethal bait (1% boric acid) *vs.* liquid non-lethal bait	*M. rubra* (12)

[a]All baits contained 1% (total) essential amino acids (glutamic acid, alanine, isoleucine, leucine, valine, tryptophan, phenylalanine, arginine, histidine, threonine, methionine; Sigma-Aldrich) and 4.55% sucrose (Sigma-Aldrich)

[b] Percentages are expressed as weight by volume (w/v)

Table 9.2. Modelled estimated marginal means and 95% confidence intervals with Bonferroni corrected pairwise comparisons between treatments (lethal *vs.* non-lethal bait) in the field experiment with *Myrmica rubra* (Fig. 9.5).

	Estimated marginal mean (confidence interval)			
Day	Lethal bait	Non-lethal bait	Z-ratio	P-value
1	9.98 (4.08 - 24.4)	24.87 (13.83 - 44.72)	2.87	0.1358
4	11.45 (6.08 - 21.580)	23.88 (15.27 - 37.35)	3.18	**0.0479**
7	6.27 (2.59 - 15.19)	17.21 (7.6 - 38.98)	2.82	0.1601
12	6.38 (2.42 - 16.82)	30.34 (19.02 - 48.4)	4.87	**<0.0001**
14	4.64 (1.52 - 14.15)	27.29 (16.69 - 44.63)	4.89	**<0.0001**
18	1.6 (0.46 - 5.54)	16.06 (8.8 - 29.29)	5.62	**<0.0001**
22	3.6 (1.39 - 9.3)	23.82 (14.64 - 38.76)	5.95	**<0.0001**
26	3.34 (1.46 - 7.66)	21.77 (13.44 - 35.26)	6.56	**<0.0001**
29	10.74 (5.62 - 20.54)	25.76 (16.73 - 39.67)	3.78	**0.0053**
33	9.2 (4.38 - 19.31)	32.13 (23.51 - 43.92)	5.22	**<0.0001**
36	40.07 (28.77 - 55.81)	53.02 (37.29 - 75.39)	1.95	1.0000
40	75.73 (54.14 - 105.92)	78.23 (57.07 - 107.22)	0.24	1.0000
44	54.69 (35.52 - 84.19)	61.95 (44.8 - 85.67)	0.78	1.0000
47	69.03 (45.97 - 103.66)	69.28 (51.63 - 92.96)	0.02	1.0000
50	43.45 (28.97 - 65.16)	62.49 (44.39 - 87.99)	2.30	0.7005
54	47.44 (33.29 - 67.59)	58.52 (44.55 - 76.87)	1.58	1.0000
57	72.28 (52.25 - 99.99)	85.19 (62.39 - 116.32)	1.23	1.0000
61	71.3 (51.51 - 98.69)	76.63 (56.58 - 103.77)	0.54	1.0000
64	66.48 (43.79 - 100.92)	76.15 (53.81 - 107.76)	0.84	1.0000
68	69.29 (51.1 - 93.94)	67.84 (47.41 - 97.06)	-0.15	1.0000
71	68.22 (50.26 - 92.6)	85.24 (63.25 - 114.89)	1.75	1.0000
75	45.98 (34.15 - 61.91)	74.79 (55.82 - 100.21)	3.92	**0.0029**
78	49.1 (36.49 - 66.06)	76.64 (58.23 - 100.87)	3.70	**0.0071**
82	41.39 (30.51 - 56.15)	83.96 (61.95 - 113.78)	5.52	**<0.0001**
89	22.84 (13.93 - 37.46)	60.49 (42.15 - 86.8)	5.34	**<0.0001**
92	23.81 (14.96 - 37.9)	69.74 (48.51 - 100.25)	6.12	**<0.0001**
96	23.2 (12.72 - 42.32)	80.06 (58.63 - 109.3)	6.15	**<0.0001**
99	33.08 (20.3 - 53.91)	78.8 (58.18 - 106.72)	5.07	**<0.0001**
103	21.92 (12.32 - 39.03)	75.67 (56.38 - 101.57)	6.43	**<0.0001**
107	25.02 (13.11 - 47.74)	78.6 (59.8 - 103.3)	5.48	**<0.0001**
110	25.58 (13.11 - 49.89)	80.95 (61.6 - 106.37)	5.37	**<0.0001**
113	19.37 (10.72 – 35)	79.97 (59.89 - 106.77)	7.24	**<0.0001**
114	14.16 (6.94 - 28.88)	74.34 (54.95 - 100.57)	7.20	**<0.0001**

9.10. Figures

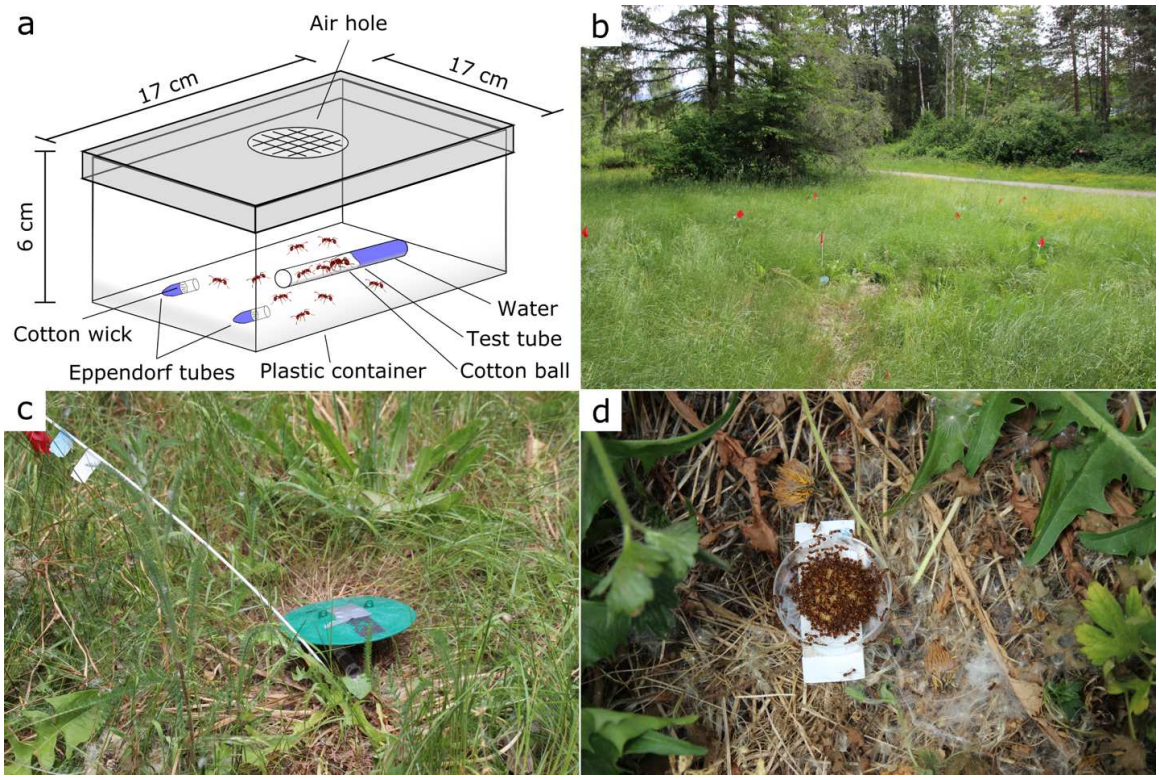


Figure 9.1. **Graphical and photographical illustrations of experimental designs. (a) Representative container used for testing bait lethality to *Myrmica rubra, Camponotus modoc*, and *Formica oreas* (modified from AR et al., unpubl.). Ants were presented with 1.5-mL Eppendorf tubes containing test baits; design details varied with experiments (see materials and methods). (b-d) Photographs of (b) the *M. rubra* field site, with red flags marking the location of ant colonies and bait stations; (c) a single bait station covered with a Unitrap lid to protect a 15-mL Falcon tube containing either a lethal or non-lethal bait; (d) apple bait in petri dish with *M. rubra* foragers; twice per week, apple baits were placed at bait stations and photographed after 70 min to record the number of foragers for analyses.**

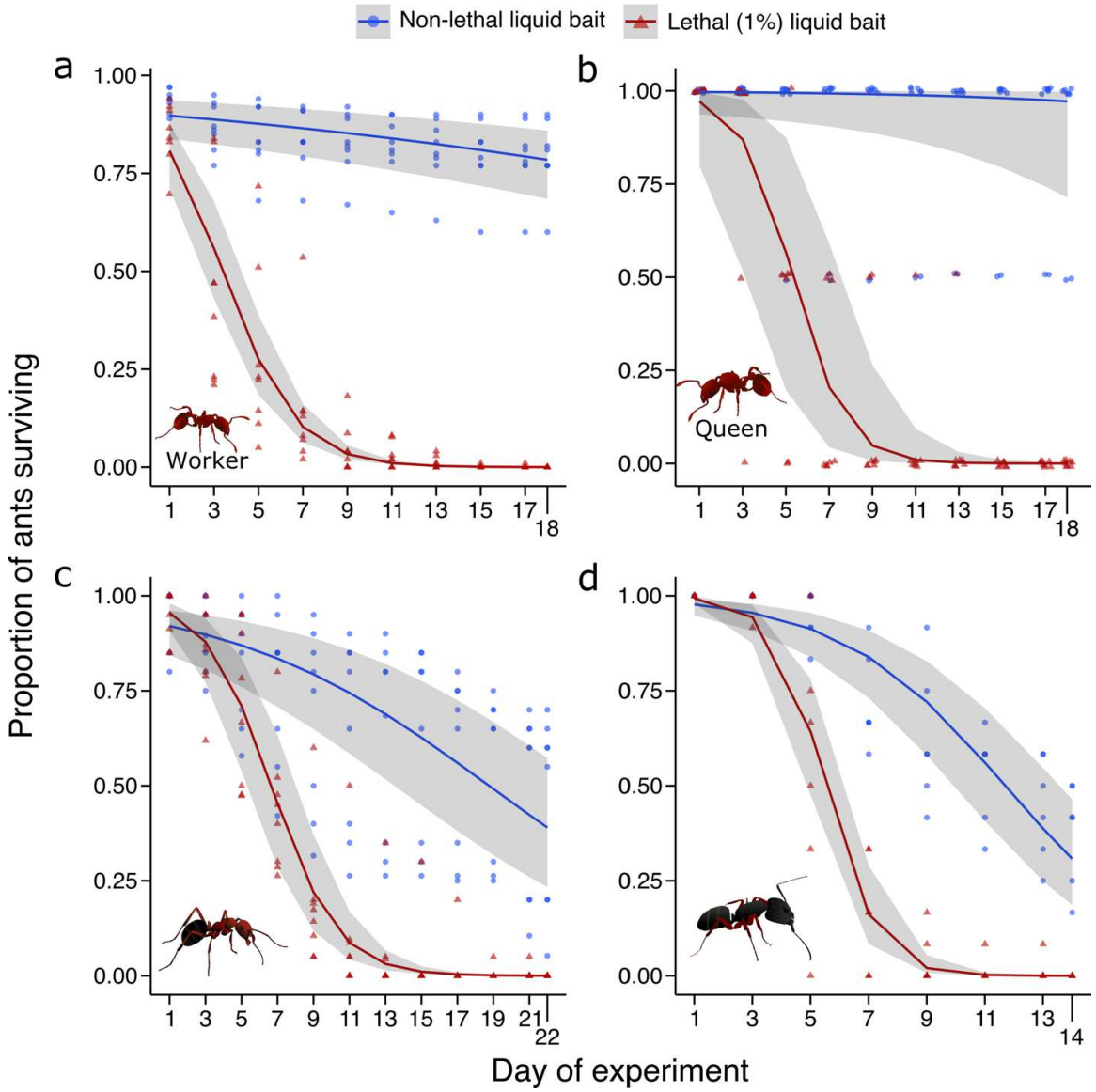


Figure 9.2. **Effect of lethal and non-lethal liquid food baits on ant survival. Treatment groups of ants were offered one lethal bait and one non-lethal bait, whereas control groups were offered two non-lethal baits. All baits contained sucrose (4.55%) and essential amino acids (1%) with boric acid (1%), or not (control), as the lethal agent. We tested colonies of (a, b) *Myrmica rubra* (n = 8), each consisting of 100 workers and two queens, (c) 20-worker groups of *Formica oreas* (n = 8), and (d) 12-worker groups of *Camponotus modoc* (n = 6). Red triangles and blue circles show the results of single replicates with lethal and non-lethal baits, respectively. Lines and shaded regions show back transformed model predicted means and 95% confidence intervals from a binomial GLMM with a logit link function. For all panels, there was a significant effect of treatment (lethal *vs.* non-lethal bait), day in experiment, and interaction between treatment and day (likelihood ratio tests; $p < 0.05$).

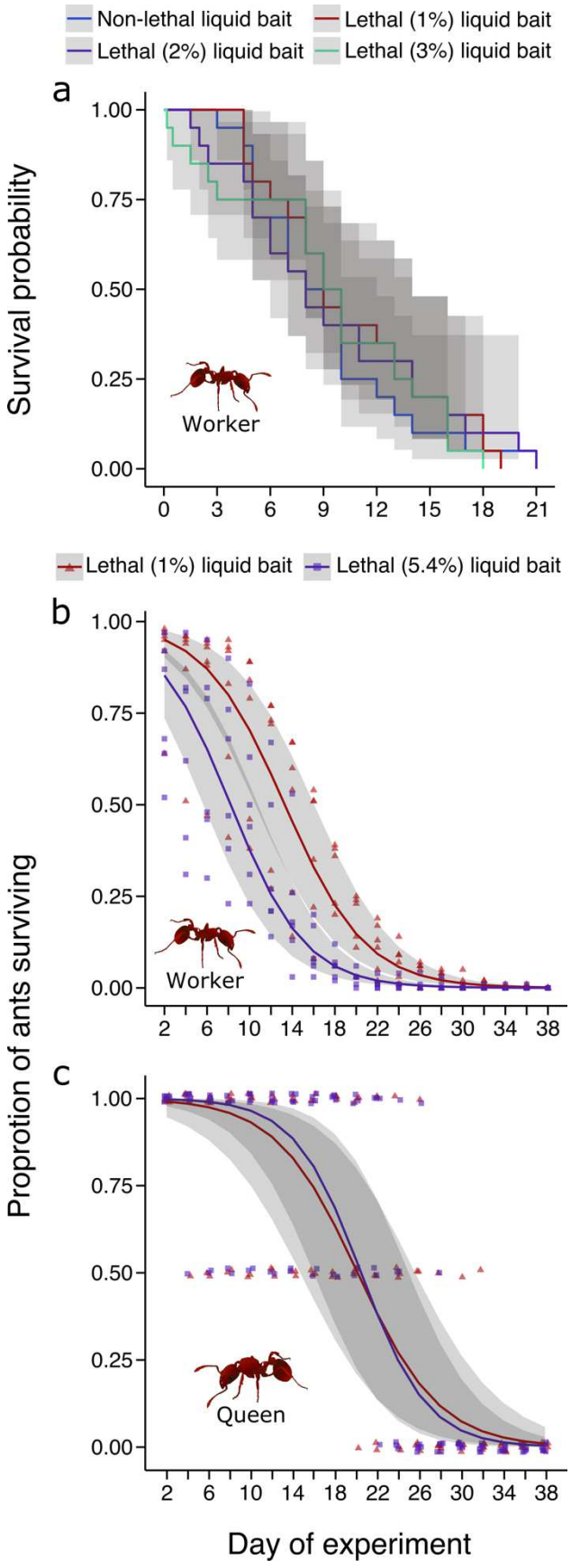

Non-lethal liquid bait
Lethal (1%) liquid bait
Lethal (2%) liquid bait
Lethal (3%) liquid bait
a
Survival probability
1.00
0.75
0.50
0.25
0.00
0
3
6
9
12
15
18
21
Worker
Lethal (1%) liquid bait
Lethal (5.4%) liquid bait
b
Proprotion of ants surviving
1.00
0.75
0.50
0.25
0.00
2
6
10
14
18
22
26
30
34
38
Worker
c
1.00
0.75
0.50
0.25
0.00
2
6
10
14
18
22
26
30
34
38
Queen
Day of experiment

Figure 9.3. **Effects of feeding regime, and boric acid concentration, on *Myrmica rubra* survival. (a) Kaplan-Meier plots illustrating the survival probability of individual *M. rubra* workers after a no-choice single feeding bout on a liquid bait (4.55% sucrose & 1% essential amino acids) containing, or not (control), boric acid (1, 2 or 3%) as the lethal agent. Lines indicate mean survival probability and shaded regions the 95% confidence intervals. There was no significant difference in survival probability across treatments (log-rank test; χ^2 = 0.4, d. f. = 3, p = 0.9. (b, c) Effect of boric acid concentration (1% or 5.4%) in liquid baits (see above) on survival of worker ants (b) and queen ants (c). Red triangles and purple squares show the results of single replicates (n = 6) with 5.4 % and 1% boric acid concentration, respectively. Lines and shaded regions are back-transformed model predictions with 95% confidence intervals from a GLMM with a logit link function. In panel b, there was a significant effect of treatment (4.5 % *vs.* 1% boric acid), day in experiment, and interaction between treatment and day; in panel c, there was a significant effect only of day in experiment (likelihood ratio tests; p < 0.05).**

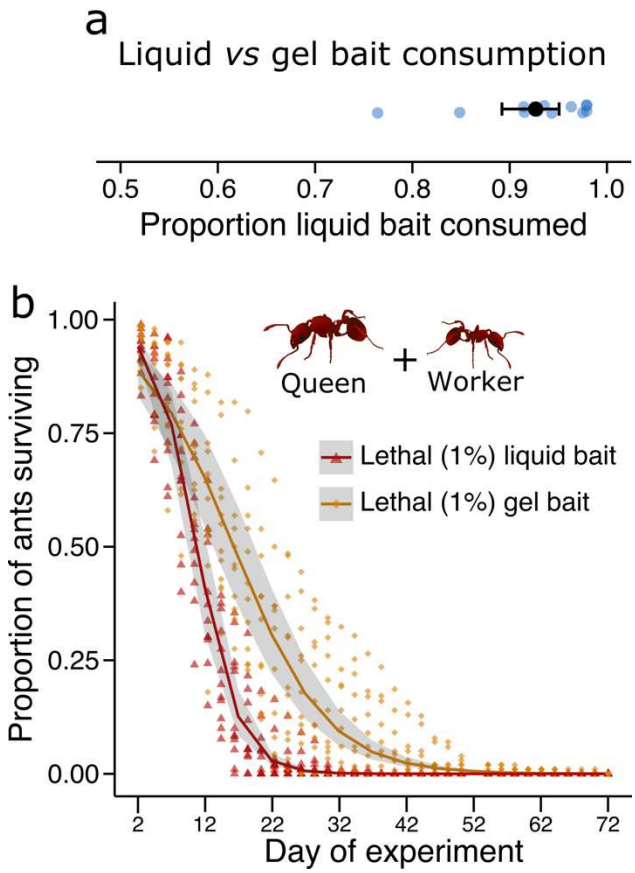


Figure 9.4. **Preferential consumption, and demise timeline, of lethal liquid and gel baits tested with laboratory colonies of *Myrmica rubra*. (a) Proportional consumption of non-lethal liquid baits (4.55% sucrose; 1% EAA) and concurrently offered non-lethal gel baits of identical nutrient composition. (b) Proportional demise of worker and queen ants in colonies feeding on either the lethal liquid bait (red, 4.55% sucrose; 1% EAA; 1% boric acid) or the lethal gel bait (orange) with identical nutrient and lethal agent composition. Blue circles, red triangles, and orange diamonds indicate the results of individual replicates (n = 12). In subpanel a, the black symbol and whiskers indicate the estimated marginal mean and 95% confidence intervals, and in subpanel b, lines and shaded regions represent back-transformed model predictions and 95% confidence intervals from a beta-binomial GLMM with a logit link function. There was a significant effect of treatment (liquid bait *vs.* gel bait), day in experiment, and interaction between treatment and day.**

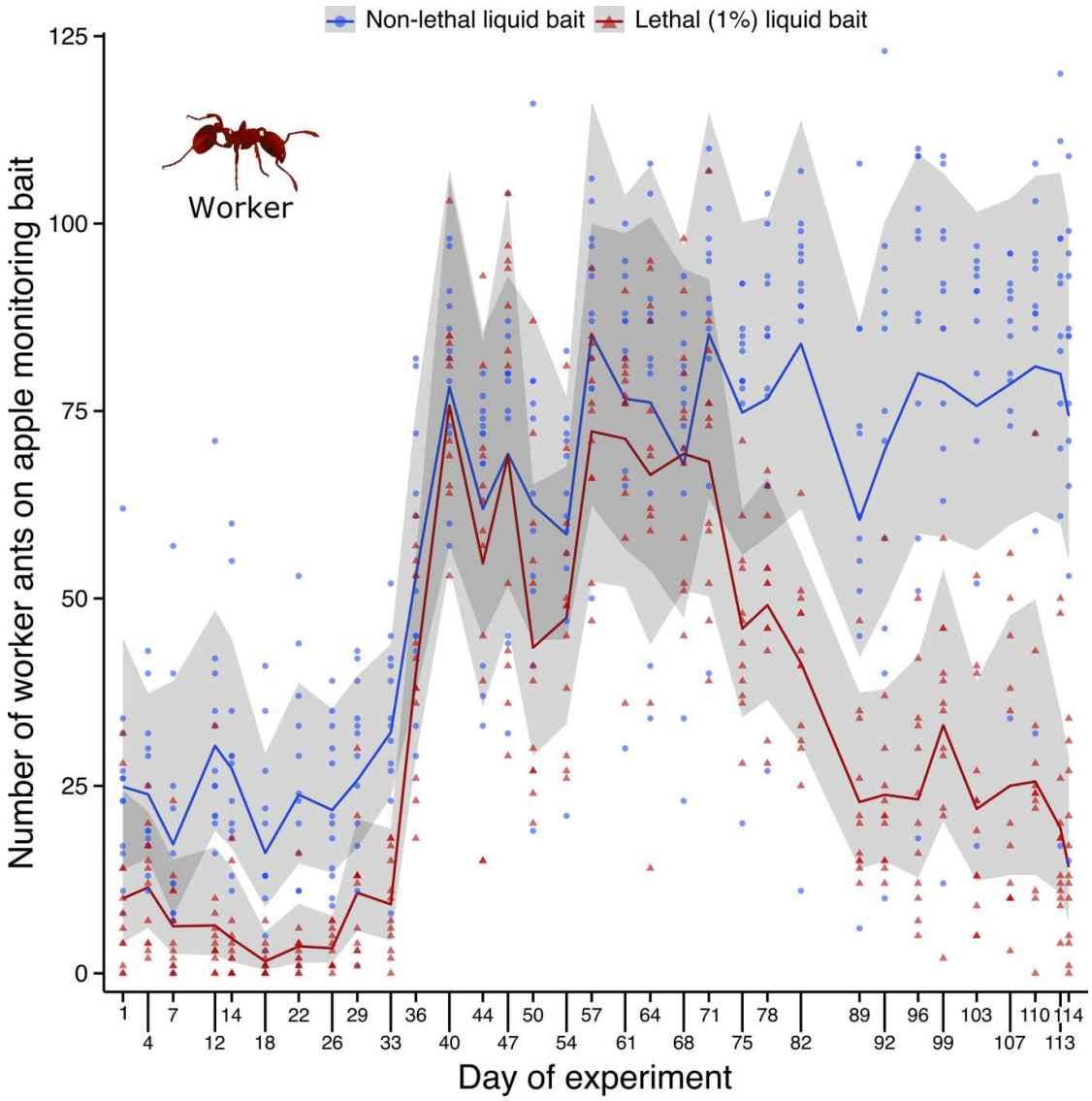


Figure 9.5. **Effect of lethal and non-lethal baits on colony demographics of *Myrmica rubra* tested at Inter River Park (North Vancouver, CA) between 23 June and 14 August 2022. Twelve colonies in the treatment plot were each provided with a lethal liquid bait (4.55% sucrose; 1% essential amino acids; 1% boric acid), and 12 colonies in the control plot were each provided with the corresponding non-lethal liquid bait lacking boric acid as the lethal agent. Colony demographics were estimated by placing apple monitoring baits twice per week for 70 min next to treatment and control colonies, and by counting the number of *M. rubra* worker ants on these baits. Lines and shaded regions are back transformed model predictions and 95% confidence intervals from a tweedie-distributed GLMM with a log link function. There was a significant effect of treatment (lethal *vs.* non-lethal bait), day in experiment, and interaction between treatment and day (likelihood ratio test; $p < 0.05$); see Table 9.2 for modelled estimated marginal means at each time point and their pair-wise comparisons.**

Appendix A.

Supplementary information for Chapter 3

Tables and figures

Table A1. Behavioural responses of *Camponotus modoc* carpenter ants before and during playback of vibratory stimuli, with back-transformed estimated marginal means (EMM) and 95% confidence intervals (CIs). We report the number of ant visits to micro-locations in a bioassay arena, where we present vibratory stimuli. The micro-location with the speaker kept silent both before and during stimulus presentation is labelled "c", and the micro-location with the speaker kept silent before, but not during, stimulus presentation id labelled "t".

Response	Before or During Stimulus	EMM	CIs
Freezing	Before	3.62	(1.5-8.73)
Freezing	During	46.01	(22.32-94.83)
Speed	Before	3.2	(2.46-4.16)
Speed	During	6.88	(5.12-9.25)
Attraction – c	Before	4.87	(3.25-7.31)
Attraction – t	Before	6.79	(4.71-9.78)
Attraction – c	During	3.4	(2.14-5.38)
Attraction – t	During	3.57	(2.27-5.61)

Table A2. **Time (s) spent frozen (no locomotion, and neither grooming nor trophallaxis behaviour) by *Camponotus modoc* worker ants 'before' and 'during' exposure to alarm signals: pheromone ("P"), vibration ("V"), or both ("P+V"), with back-transformed estimated marginal means (EMM) and 95% confidence intervals (CIs). We also report Tukey adjusted pairwise comparisons between 'before' and 'during' time periods within an alarm signal, and across alarm signals in the 'during' time interval.**

Alarm signal	Before or During alarm signal	EMM	CIs	Contrast	Nested variable	Z ratio	P value
P	Before	2.36	(0.8-6.99)	Before *vs* During	P	-1.93	0.05
P	During	6.07	(2.34-15.73)	Before *vs* During	V	-5.1	<0.0001
V	Before	2.16	(0.76-6.16)	Before *vs* During	P+V	-6.93	<0.0001
V	During	19.33	(8.83-42.31)	P *vs* V	During	-2.55	0.03
P+V	Before	2.62	(0.96-7.18)	P *vs* P+V	During	-4.44	<0.0001
P+V	During	42.53	(20.63-87.67)	V *vs* P+V	During	-2.04	0.1

Table A3. **Running speed (cm/s) of *Camponotus modoc* worker ants 'before' and 'during' alarm signals: pheromone ("P"), vibration ("V") or both ("P+V"), with back-transformed estimated marginal means (EMM) and 95% confidence intervals (CIs). We also report Tukey adjusted pairwise comparisons between 'before' and 'during' time intervals within an alarm signal, and across signals, in the 'during' time interval.**

Alarm signal	Before or during alarm signal	EMM	CIs	Contrast	Nested variable	Z ratio	P value
P	Before	5.15	(4.31-6.16)	Before *vs* During	P	-1.13	0.26
P	During	5.66	(4.73-6.77)	Before *vs* During	V	-7.34	<0.0001
V	Before	5.66	(4.78-6.71)	Before *vs* During	P+V	-5.18	<0.0001
V	During	10.1	(8.52-11.96)	P *vs* V	During	-6.16	<0.0001
P+V	Before	5.55	(4.69-6.58)	P *vs* P+V	During	-4.15	<0.0001
P+V	During	8.36	(7.05-9.9)	V *vs* P+V	During	2.07	0.1

Table A4. **Number of visits by *Camponotus modoc* worker ants 'before' and 'during' exposure to alarm signals: alarm pheromone ("P"), vibration ("V") or both ("P+V"), with back-transformed estimated marginal means (EMM) and 95% confidence intervals (CIs). We report the number of visits to micro-locations (ML) in a bioassay arena, where we present alarm pheromone, vibratory signals, both or signal. The micro-location without any alarm signal 'before' and 'during' signal representation is labeled "c", whereas the micro-location with no signal in the 'before' time interval but with a signal in the 'during' time interval is labelled "t". We also report Tukey adjusted pairwise comparisons between speakers within the 'before' and the 'during' time intervals, and within a signal and across signals in the 'during' time interval (Note: nested variables (NV) 1 and 2 represent the variables within which pairwise comparisons are made).**

Alarm signal	Before or During signal	ML	EMM	CIs	Contrast	NV1	NV2	Z ratio	P value
P	Before	c	4.73	(2.83-7.91)	c *vs* t	P	Before	-1.99	0.05
P	Before	t	7.04	(4.33-11.46)	c *vs* t	P	During	-5.7	<0.0001
P	During	c	7.05	(4.33-11.48)	c *vs* t	V	Before	1.47	0.14
P	During	t	20.35	(13.5-30.69)	c *vs* t	V	During	-2.08	0.04
V	Before	c	6.96	(4.34-11.17)	c *vs* t	P+V	Before	-0.85	0.4
V	Before	t	5.25	(3.26-8.45)	c *vs* t	P+V	During	-4.39	<0.0001
V	During	c	7.37	(4.71-11.55)	P *vs* V	t	During	3.12	0.01
V	During	t	10.77	(6.95-16.71)	P *vs* P+V	t	During	2.71	0.02
P+V	Before	c	5.83	(3.64-9.35)	V *vs* P+V	t	During	-0.47	0.89
P+V	Before	t	6.86	(4.3-10.96)					
P+V	During	c	5.2	(3.17-8.53)					
P+V	During	t	11.85	(7.8-18.01)					

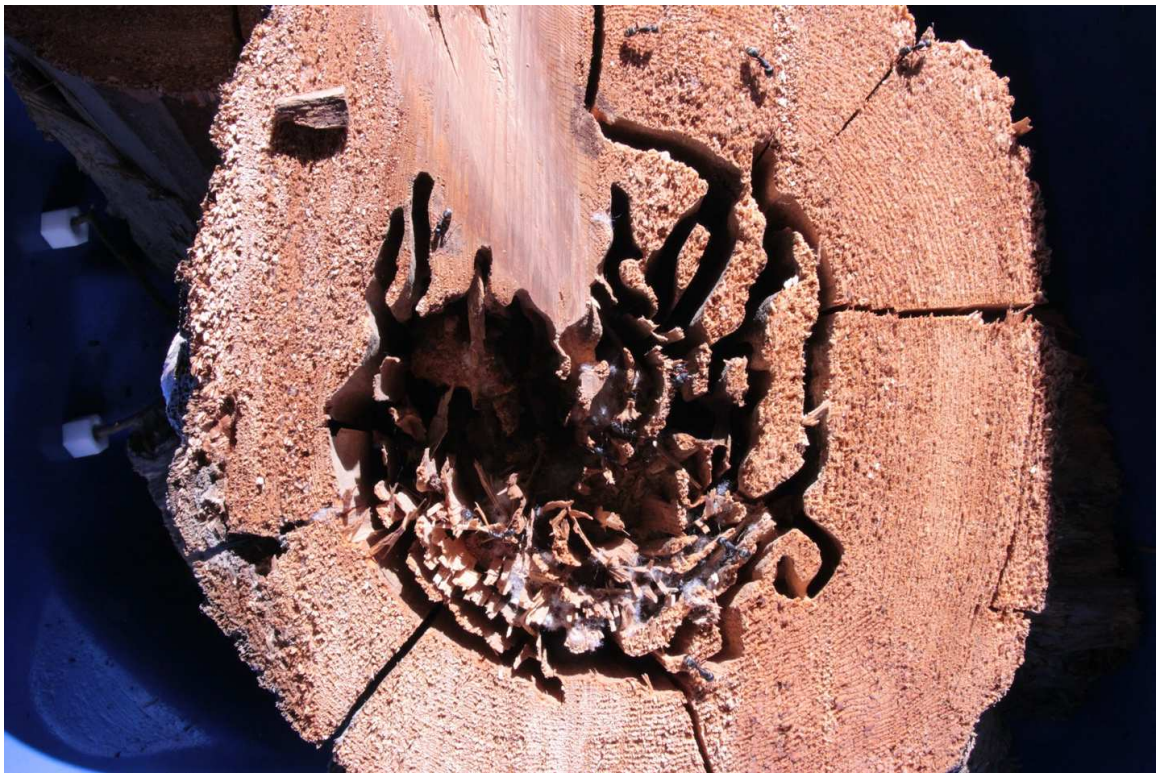


Figure A1. **Cross section of a *Camponotus modoc* nest revealing sculpting of wood tunnels and lamellae.**

Appendix B.

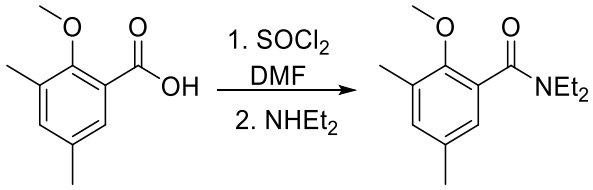
Supplementary information for Chapter 5

Chemical synthesis

Synthesis of 8-hydroxy-3,5,7-trimethylisocomarin (5)

The synthesis proceeded in four steps, as follows:

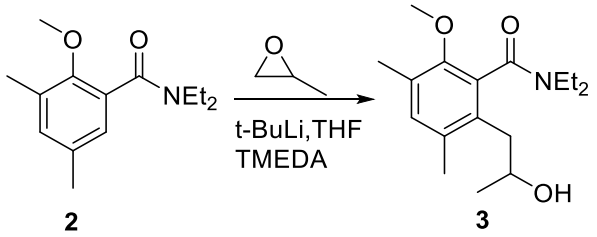
Step 1: *N*,*N*-Diethyl-2-methoxy-3,5-dimethylbenzamide (2)



1 **2**

Neat thionyl chloride (8.68 ml, 0.119 mol) was added to 2-methoxy-3,5-dimethylbenzoic acid (**1**) (1.57 g, 8.32 mmol) and stirred under argon. After *N,N*-dimethylformamide (213 µL, 0.275 mmol) was added dropwise (which resulted in vigorous effervescence), the solution was stirred for 1 additional hour. Excess thionyl chloride was removed azeotropically with toluene (3 × 10 mL) under reduced pressure. The colourless oily residue thus obtained was dissolved in anhydrous tetrahydrofuran (THF) (10 mL) and cooled to 0 °C after which diethylamine (3.184 ml, 30.78 mmol) was added slowly. The mixture was then stirred 15 min and concentrated. The crude residue was dissolved in CH_2Cl_2 (40 mL), washed with water (3 × 10 mL) and brine (3 × 10 mL), and then concentrated under reduced pressure to afford a crude brown oil. Following removal of impurities, *N*,*N*-diethyl-2-methoxy-3,5-dimethylbenzamide (**2**) (1.93 g, 99 %) was obtained as a brown oil. Spectral data of **2** matched those reported in the literature (Bestmann et al. 1992).

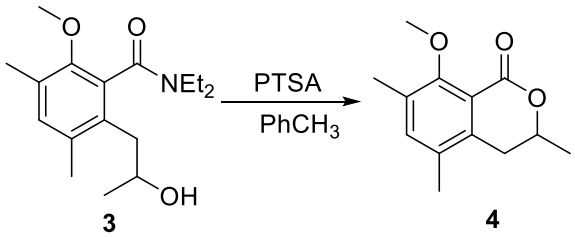
Step 2: N,N-diethyl-2-(2-hydroxypropyl)-6-methoxy-3,5-dimethylbenzamide (3)



2 **3**

To a solution of **2** (1 g, 4.24 mmol) in dry THF (10 mL) tetramethylethylenediamine (TMEDA) (1.39 mL, 9.3 mmol) was added at -78 °C. A solution of *t*-BuLi in hexane (2.5M, 3.75 mL, 9.3 mmol) was then added dropwise at -78 °C. After 2 h, propylene oxide (0.355 mL, 5.08 mmol) was added dropwise at -78 °C. After stirring the reaction mixture at -78 °C for 7 h, it was quenched with saturated aqueous NH_4Cl and aqueous 1M HCl, and extracted with EtOAc and CH_2Cl_2. The combined extracts were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel chromatography to afford *N*,*N*-diethyl-2-(2-hydroxypropyl)-6-methoxy-3,5-dimethylbenzamide (**3**) (621 mg, 50%). Spectral data of **3** matched those reported in the literature (Bestmann et al. 1992).

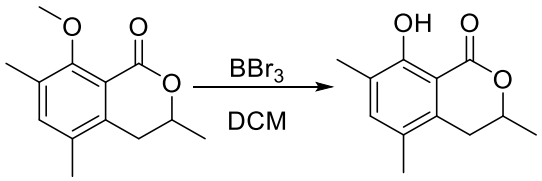
Step 3: 8-Methoxy-3,5,7-trimethylisocoumarin (4)



3 **4**

To a solution of **3** (360.4 mg, 1.23 mmol) in toluene (18.0 mL) *p*-toluenesulfonic acid (*p*-TsOH) · H_2O (264.1 mg, 1.39 mmol) was added. The reaction mixture was refluxed and stirred 2 h after which it was concentrated *in vacuo*. The residue was purified by silica gel chromatography (hexane: AcOEt = 1: 1) to afford **4** (189.0 mg, 70%) as a white solid. Spectral data matched those reported in the literature (Bestmann et al. 1992).

Step 4: 8-Hydroxy-3,5,7-trimethylisocoumarin (5)



4 **5**

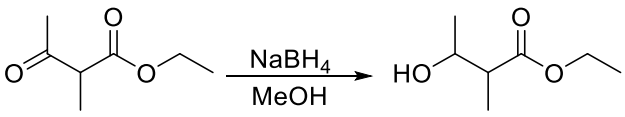
To a solution of **4** (137 mg, 0.622 mmol) in dry CH_2Cl_2 (7 mL) was added dropwise a solution of boron tribromide in CH_2Cl_2 (1.0 M, 1.5 mL, 1.5 mmol) at -78 °C. After 1 h, the temperature of the reaction mixture was raised to 0 °C and the mixture was stirred 1 h. Subsequently, the mixture was quenched with saturated aqueous NH_4Cl, and extracted with CH_2Cl_2. Combined extracts were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (hexane: AcOEt = 3: 1) to afford **5** (115 mg, 90%) as white solid. Spectral data matched those reported in the literature (Bestmann et al. 1992).

Reference

Bestmann HJ, Kern F, Schäfer D, Witschel MC (1992) 3,4-Dihydroisocoumarins, a new class of ant trail pheromones. *Angew Chem Int Ed 31*, 795.

Synthesis of racemic 2,4-dimethyl-5-hexanolide (3,5,6-trimethyltetrahydro-2*H*-pyran-2-one) (14)

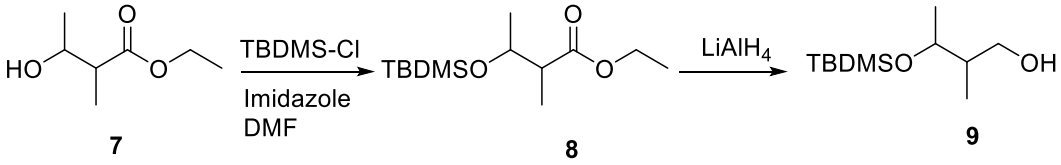
Ethyl-3-hydroxy-2-methylbutanoate (7)



6 **7**

Ethyl 2-methyl-3-oxobutanoate (**6)** (2 g, 13.8 mmol) was dissolved in methanol (40 mL) and sodium borohydride (0.52 g, 13.8 mmol) was added. After keeping the reaction mixture at room temperature (rt) for 1 h, methanol was evaporated, and the synthetic product extracted with diethyl ether and dried over $MgSO_4$. Following solvent evaporation and residue distillation by Kugelrohr (b.p. 70 ± 80 °C), ethyl-3-hydroxy-2-methylbutanoate (**7**) (1.88 g; 50% diastereoisomers) was obtained as a colorless oil. Spectral data of **7** matched those reported in the literature (Bestmann et al., 1999).

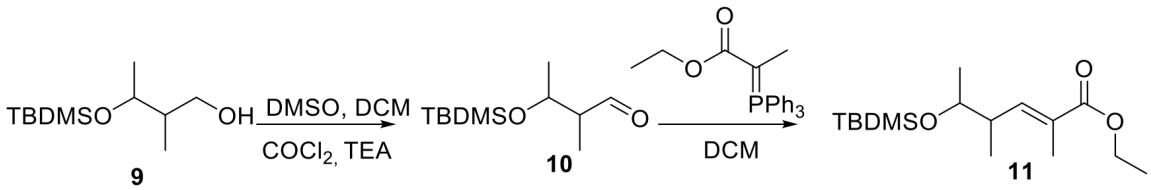
3-((Tert-butyldimethylsilyl)oxy)-2-methylbutan-1-ol (9)



7 **8** **9**

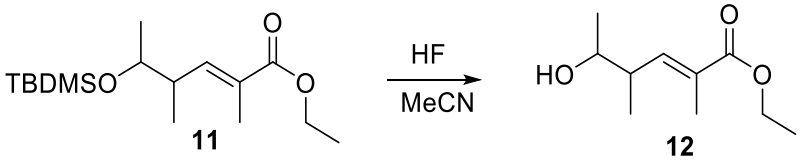
To a solution of imidazole (470 mg, 6.9 mmol) in dimethylformamide (DMF) was added tert-butyldimethylsilyl chloride (550 mg, 3.45mmol). After 20 min, a solution of **7** (400 mg, 2.75 mmol) in DMF (2 mL) was added, with stirring continuing for 24 h at room temperature. The mixture was quenched with a dilute aqueous solution of NaCl, extracted (3 ×) with diethyl ether, and dried over $MgSO_4$. The solvent was removed *in vacuo* and the remaining oil passed through silica (10 g) and eluted with pentane/AcOEt (5:1) to yield the silylated hydroxyl ester **8**, which was taken up in toluene and added to a suspension of $LiBH_4$ (75 mg, 3.45 mmol) in diethyl ether (6 mL). After 5 h, the temperature was gradually increased, solvents were removed under reduced pressure, and the solid residue was hydrolyzed with dilute aqueous HCl. When the solution had reached a pH of 6, it was saturated with K_2CO_3 and extracted (3 ×) with diethyl ether. After drying over $MgSO_4$, the solvent was removed *in vacuo* and the crude product purified by chromatography (petroleum ether/ethyl acetate (5/1)), yielding 3-((tert-butyldimethylsilyl)oxy)-2-methylbutan-1-ol (**9)** (370 mg (88%)) as a colorless oil. Spectral dataof **9** matched those reported in the literature (Bestmann et al., 1999).

Ethyl-(*E*)-5-((tert-butyldimethylsilyl)oxy)-2,4-dimethylhex-2-enoate (11)



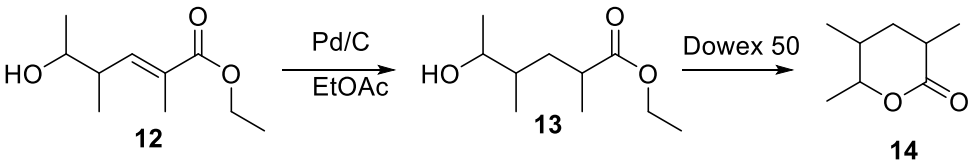
To a stirred solution of oxalyl chloride (0.215 mL, 2.5 mmol) in CH_2Cl_2 (15 mL) at -78 °C was added a solution of dimethyl sulfoxide (DMSO) (0.265 mL, 3.75 mmol) in CH_2Cl_2 (5 mL). After 10 min, a solution of 3-((tert-butyldimethylsilyl)oxy)-2-methylbutan-1-ol (**9**) (260 mg, 1.2 mmol) in CH_2Cl_2 (6 mL) was added, and the mixture was stirred 30 min at -78 °C. After slowly adding triethylamine (1.6 mL, 11 mmol) s and the mixture had warmed to 0 °C, (carboethoxyethylene)triphenylphosphorane (500 mg, 1.45 mmol) was added, and the mixture was stirred and heated (45 °C) under reflux overnight. The solvent was evaporated *in vacuo* and the residue diluted with diethyl ether, hydrolyzed with water, and extracted with diethyl ether. After drying over $MgSO_4$ and concentrating *in vacuo*, the product was purified by chromatography on silica gel with pentane/ethyl acetate (5/1) to yield ethyl (*E*)-5-((tert-butyldimethylsilyl)oxy)-2,4-dimethylhex-2-enoate (**11)** (255 mg, 71%) as a colorless oil. Spectral data of **11** matched those reported in the literature (Bestmann et al., 1999).

Ethyl (E)-5-hydroxy-2,4-dimethylhex-2-enoate (12)



To a solution of **11** (90 mg, 0.3 mmol) in CH_3CN was added HF (1 mL, 50% aqueous solution). After stirring the mixture 2 h at rt , it was diluted with diethyl ether (10 mL), extracted (2 ×) with aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the crude product purified by chromatography on silica gel with pentane/ethyl acetate to yield ethyl (*E*)-5-hydroxy-2,4-dimethylhex-2-enoate (**12**) **(**58 mg, 88%) as a colorless oil.

2,4-Dimethyl-5-hexanolide (3,5,6-trimethyltetrahydro-2H-pyran-2-one) (14)



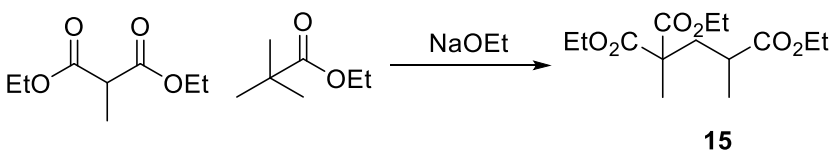
A solution of compound **12** (50 mg) in diethyl ether was hydrogenated with Pd/C/10% catalyst. After the solvent was distilled and the residue dissolved in THF (15 mL), Dowex 50 (10 mg) was added, and the suspension stirred 30 min. The solvent was evaporated and the residue chromatographed on silica gel with ether as eluent, affording a mixture of 2,4-dimethyl-5-hexanolides (3,5,6-trimethyltetrahydro-2*H*-pyran-2-one) (**14**) (23 mg, 60 % yield).

Reference

Bestmann HJ, Liepold B, Kress A, Hofmann A (1999) (2*S*,4*R*,5*S*)-2,4-Dimethyl-5-hexanolide: Ants of different species *Camponotus* can distinguish the absolute configuration of their trail pheromone**.** *Chem Eur J 5*, 2984.

Synthesis of (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide (25)

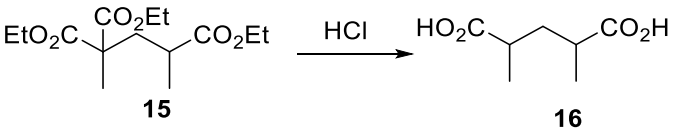
2-Ethoxycarbonyl-2,4-dimethyl-pentanedioic acid diethyl ester (15)



Sodium metal (540 mg, 23.5 mmol) was added in small pieces to 15 mL of ethanol. After all the sodium had reacted, the solution was heated to reflux and diethyl methylmalonate (4.05 mL, 23.5 mmol) and then ethyl 2-methyl-2-bromopropionate (3.45

mL, 23.5 mmol) were added. After stirring the reaction mixture 2 h at reflux, it was cooled and most of the ethanol removed in *vacuo*. Water (7.5 mL) was added and the resulting aqueous layer extracted with ether. The combined organics were washed with brine, dried over anhydrous $MgSO_4$, and then concentrated. The crude product was purified by distillation at reduced pressure (85-110ºC, 2.5 mmHg) to afford compound **15** (4.75 g, 70%) as a colourless oil.

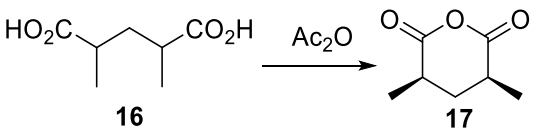
2,4-Dimethyl-pentanedioic acid (16)



15 **16**

A solution of the tri-ester **15** (4.3 g, 15 mmol) in concentrated HCl (12.5 mL) was heated to reflux 24 h. After the solution had cooled to room temperature, it was stored 20 h at 0 ºC. The crystals that had formed were filtered out, dissolved in diethylether and dried over anhydrous $MgSO_4$, affording - after ether removal – compound **16** (1.6 g) as white crystals. The aqueous HCl layer was then extracted with ether (5 × 10 mL). The combined ether layers were dried over anhydrous $MgSO_4$ and concentrated to give an oily sludge which was recrystallized from pentanes to afford more of compound **16** (500 mg, combined yield of 2.1 g, 88%).

3,5-Dimethyl-dihydro-pyran-2,6-dione (17)

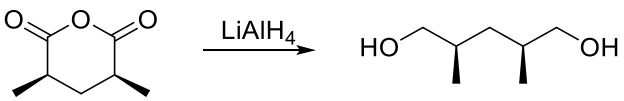


16 **17**

A solution of the diacid **16** (2 g, 1.25 mmol) in acetic anhydride (5 mL) was refluxed 4 h. The acetic anhydride and acetic acid were then distilled off and the crude product was purified by Kugelrohr distillation (80-120 ºC, 0.2 mm Hg) to give a mixture of

meso and dl anhydrides. The product was recrystallized from ethyl acetate (5-10 mL), affording the meso product **17** (444 mg, 25%) as white crystals.

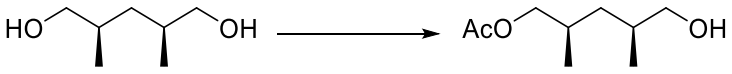
Meso-2,4-dimethylpentane-1,5-diol (18)



17 **18**

To a solution of the meso-2,4-dimethylglutaric anhydride **17** (400 mg, 2.81 mmol) in anhydrous THF (10 mL), lithium aluminum hydride (320 mg, 8.44 mmol) was added in four equal portions at 0 °C. The reaction mixture was warmed to and kept at room temperature 5 min and then heated (80 °C) and refluxed 24 h. The reaction mixture was cooled to 0 °C and excess hydride cautiously quenched by the sequential addition of water (5 mL) and hydrochloric acid (6N; 3 mL). The resulting white suspension was warmed to room temperature and the aqueous layer extracted with ethyl acetate (6 × 40 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure, yielding the desired diol **18** (390 mg; 98%) as a colorless oil.

(2*S*,4*R*)-5-Hydroxy-2,4-dimethylpentyl acetate (19)

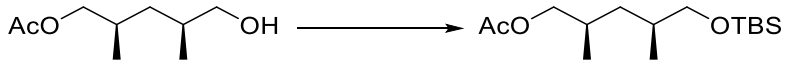


18 **19**

To a solution of the *meso*-diol **18** (350 mg, 2.67 mmol) and vinyl acetate (238 mg, 0.25 mL, 2.75 mmol) in THF (10 mL) was added Amano Lipase AK from *Pseudomonas fluorescence*(10 mg) (Aldrich Cat. Nr. 53,473-0), and the resulting suspension was stirred at room temperature. After 24 h, additional vinyl acetate (0.05 mL) was added, and stirring continued for a total of 80 h. The reaction mixture was then filtered through a Celite pad (2 cm), and the filtrate concentrated *in vacuo*. The residue

was re-dissolved in CH_2Cl_2 (10 mL). This solution was washed with a 15% NaCl water solution (2 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo* to yield a mixture of the mono-acetate **19** and its corresponding diacetate (430 mg, 85/15 ratio) as a slightly yellow oil. The overall yield of **19** was 80%.

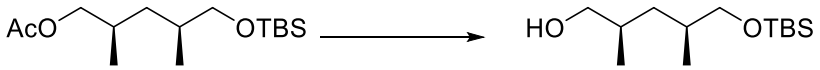
(2*S*,4*R*)-5-{[(1,1-Dimethylethyl)(dimethyl)silyl]oxy}-2,4-dimethylpentyl acetate (20)



19 **20**

To a solution of the acetate mixture (**19**; 400 mg) and imidazole (280 mg, 4 mmol) in CH_2Cl_2 (10 mL) was added over 15 min *tert*-butyldimethylchlorsilane (300 mg, 2 mmol) in small portions, stirring the reaction mixture for further 40 min. The imidazole hydrochloride was filtered and the filtrate washed with water (2 × 10 mL), 3% HCl (2 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 10 mL) and brine (1 × 10 mL), and then dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give the crude product **20** (600 mg; 98%) as a colorless oil, which was used for the next step without further purification.

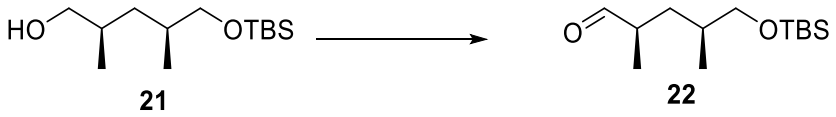
(2*S*,4*R*)-5-{[(1,1-Dimethylethyl)(dimethyl)silyl]oxy}-2,4-dimethylpentan-1-ol (21)



20 **21**

To a solution of the crude silyl ether **20** (600 mg) from the previous step in MeOH (5 mL) was added powdered K_2CO_3 (336 mg, 2.4 mmol) stirring the mixture 2 h. Thereafter, the solids were removed by filtration and the filtrate concentrated *in vacuo*. The residue was re-dissolved in petroleum ether (10 mL), washed with water (3 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and concentrated *in vacuo* affording alcohol **21** (420 mg) as a colorless oil.

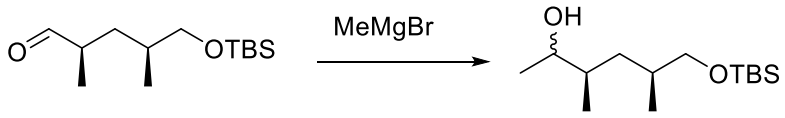
(2*S*,4*R*)-5-{[(1,1-Dimethylethyl)(dimethyl)silyl]oxy}-2,4-dimethylpentanal (22)



21 **22**

To a stirred solution of alcohol **21** (370 mg, 1.5 mmol) in CH_2Cl_2 (8 mL) a mixture of iodobenzene diacetate (724 mg, 2.25 mmol) and TEMPO (11.7 mg, 0.075 mmol) was added in five portions over 30 min. The orange reaction mixture was stirred for further 60 min at 23 °C before a 25% solution of sodium thiosulphate (10 mL) was added. The mixture was stirred 15 min and the organic phase separated. The aqueous phase was extracted with CH_2Cl_2 (5 mL), and the combined organic layers were washed with a saturated aqueous $NaHCO_3$ solution (2 × 10 mL) and brine. The organic phase was dried over Na_2SO_4, and filtered through a short pad of silica gel (2 cm), followed by washing the pad with CH_2Cl_2 (2 × 10 mL), affording the "oily" aldehyde **22** (350 g, 96% yield).

(3*R*,5*S*)-6-((*tert*-Butyldimethylsilyl)oxy)-3,5-dimethylhexan-2-ol (23)

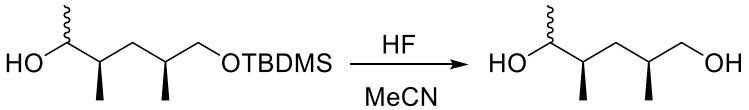


22 **23**

A solution of aldehyde **22** (300 mg, 1.2 mmol) in anhydrous tetrahydrofuran (7 mL) was cooled to -20 °C before a solution of methylmagnesium chloride (2.4 mmol) in anhydrous tetrahydrofuran (2 mL) was slowly added via syringe. The reaction mixture was stirred 30 min, and saturated ammonium chloride (5 mL) was added. The organic phase was separated and the aqueous phase extracted with ethyl acetate (3 × 10 mL). Extracts were combined, dried over $MgSO_4$, the drying agent filtered off, and the solution concentrated *in vacuo*. Flash chromatography of the residue afforded the

alcohol **23** (218 mg, 70% yield) as a mixture of diastereomers (1:1 ratio) which was used in the next synthetic step.

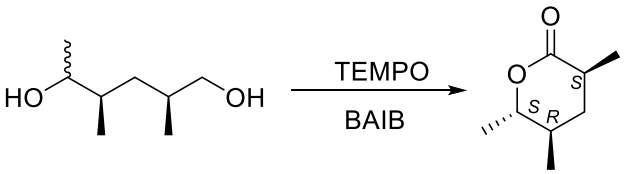
(2*S*,4*R*)-2,4-Dimethylhexane-1,5-diol (24)



23 **24**

To a solution of alcohol **23** (200 mg, 0.77 mmol) in CH_3CN was added HF (1 mL, 50% aqueous solution). After stirring the mixture 2 h at room temperature, it was diluted with diethyl ether (10 mL), extracted with aqueous $NaHCO_3$ (2 ×) and brine, and dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the crude product purified by chromatography on silica gel with pentane/ethyl acetate as eluents yielding (2*S*,4*R*)-2,4-dimethylhexane-1,5-diol **24 (**100 mg, 89% yield) as a colorless oil.

(2*S*,4*R*,5*S*)-2,4-Dimethyl-5-hexanolide (25)



24 **25**

To a stirred room-temperature (rt) solution of diol **24** (90 mg, 0.61 mmol) in CH_2Cl_2 (10 mL) was added sequentially bis-acetoxyiodobenzene (BAIB, 621 mg, 1.93 mmol) and 2,2,6,6- tetramethylpiperidinooxy (TEMPO, 18 mg, 0.12 mmol). After stirring at rt 3.5 h, saturated aqueous $Na_2S_2O_3$ and diethyl ether (5 mL) were added. The separated organic phase was washed with saturated aqueous $NaHCO_3$ and then with H_2O. The combined aqueous washes were extracted with diethyl ether (3 × 10 mL), and the combined organic fractions were washed with brine, dried ($NaSO_4$), filtered and concentrated by rotary evaporation. Purification of the residue by flash column

chromatography afforded (2*S,*4*R*,5S)-2,4-dimethyl-5-hexanolide and (2*S*,4*R*,5*R*)-2,4-dimethyl-5-hexanolide as a mixture of diastereomers. These diastereomers were separated by column chromatography (hexanes:ethyl acetate, 60:40), yielding 20 mg of (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide. Spectral data matched those reported in the literature (Bestmann et al. 1999).

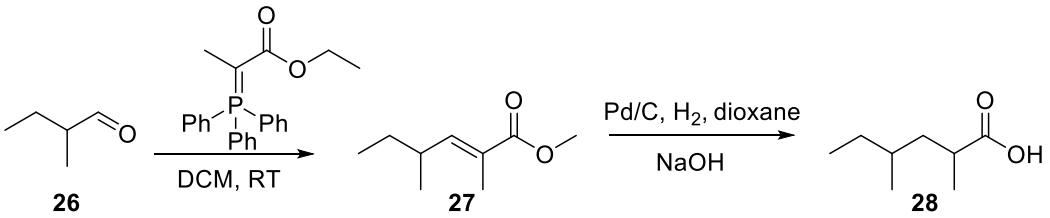
References

Zhai H, Hrabar M, Gries R, Gries G, Britton R (2016) Synthesis, stereochemical assignment, and field-testing of the sex pheromone of the strepsipteran *Xenos peckii*. *Chem Eur J 22***,** 6190.

Prusov E, Röhm H, Maier ME (2006) Chemoenzymatic synthesis of the C10−C23 segment of Dictyostatin. *Org Lett 8*, 1025.

Hansen TM, Florence GJ, Lugo-Mas P, Chen J, Abrams JN, Forsyth CJ (2003) Highly chemoselective oxidation of 1,5-diols to δ-lactones with TEMPO/BAIB. *Tetrahedron Let 44*, 57.

Synthesis of 2,4-dimethylhexanoic acid (28)



A mixture of 2-methylbutyraldehyde (172 mg, 2 mmol) and (carbethoxyethylidene)triphenylphosphorane (724 mg, 2 mmol) in DCM (5 mL) was stirred overnight at rt. After the reaction was complete, the mixture was purified by column chromatography (ether/pentane = 1/15), affording (*E*,*Z*)-2,4-dimethylhex-2-enoate (**27)** (218 mg, 70%) as a pale yellow gum. The ester **27** (200 mg, 1.28 mmol) was then dissolved in dioxane (2 ml) and 10% palladium on activated carbon (30 mg)

was added The slurry was hydrogenated 2 h under pressure using an H_2-filled balloon. The activated carbon was filtered off using celite and the filter was washed with dioxane (2 ml). After adding1N NaOH (2 ml), the mixture was left overnight at rt. After adding 1N hydrochloric acid (3 ml), the product was extracted with ethyl acetate, the organic solution dried over sodium sulfate, filtered, and the solvent removed under reduced pressure, obtaining 2,4-dimethylhexanoic acid (129 mg, 70%) as a colorless oil. Spectral data of **28** matched those reported in the literature (Leonard et al. 2002).

Reference

Leonard WR, Belyk KM, Bender DR, Conlon DA, Hughes DL, Reider P J (2002) Determination of the relative and absolute configuration of the dimethylmyristoyl side chain of pneumocandin B_0 by asymmetric synthesis. *Org Let 4*, 4201.

Appendix C.

Supplementary information for Chapter 6

Tables

Table C1. List of supplier of sugars identified in *Cinara splendens* honeydew and tested in watery solution for consumption by Western carpenter ants, *Camponotus modoc,* and European fire ants, *Myrmica rubra.*

Sugar	Supplier
Monosaccharides	
D-(+)-xylose	BDH Chemicals (part of Merck Chemicals Ltd.)
D-(-)-fructose	Sigma-Aldrich, St. Louis, MO 63118, USA
D-(+)-glucose	EMD Chemicals Inc., NJ 08027, USA
Disaccharides	
D-(+)-sucrose	Sigma-Aldrich
D-(+)-maltose monohydrate	TCI America, Portland, OR 97203, USA
D-(+)-trehalose dihydrate	TCI America
D-(+)-melibiose	Sigma-Aldrich
D-(+)-turanose	Sigma-Aldrich
Trisaccharides	
D-(+)-raffinose pentahydrate	TCI America
D-(+)-melizitose	Sigma-Aldrich
Erlose	Santa Cruz Biotechnology, Dallas, TX 75220, USA

Table C2. Results of one sample T-tests which were run to determine whether mean consumptions of sugar solutions by *C. modoc* and *M. rubra* colonies were statistically lower than zero.

Treatment	T	Df	P
Exp. 1: Worker ants of *C. modoc* seek aphid honeydew based solely on the presence of sugar constituents			
Honeydew	4.4323	5	0.9966
Synthetic blend (SB)	3.5588	5	0.9919
Exp. 2: Worker ants of *C. modoc* prefer sugar solutions containing aphid-derived sugars			
SB	2.7065	4	0.9731
SB minus raffinose	4.0579	4	0.9923
Exp 3: Worker ants of *C. modoc* preferentially seek sugar solutions with higher sugar content			
5%	3.9144	3	0.9852
20%	2.8922	3	0.9686
40%	5.5366	3	0.9942
70%	4.7946	3	0.9914
Exp. 4: Choices between monosaccharides – *C. modoc*			
Glucose	3.4424	5	0.9908
Xylose	1.6831	5	0.9234
Fructose	2.239	5	0.9623
Exp. 4: Choices between monosaccharides – *M. rubra*			
Glucose	2.6907	5	0.9784
Xylose	-0.1158	5	0.4562
Fructose	3.7863	5	0.9936
Water	-0.80471	5	0.2288
Exp. 5: Choices between disaccharides – *C. modoc*			
Melibiose	1.4297	4	0.887
Maltose	2.4894	4	0.9662
Trehalose	17.349	4	1
Sucrose	6.5137	4	0.9986
Exp. 5: Choices between disaccharides – *M. rubra*			
Melibiose	-7.3154	5	0.000374

Maltose	0.4419	5	0.6615
Trehalose	-2.8078	5	0.01882
Sucrose	4.6714	5	0.9973
Water	-4.1553	5	0.004432
Exp. 6: Choices between trisaccharides – *C. modoc*			
Melezitose	3.2997	5	0.9893
Raffinose	2.9721	5	0.9845
Exp. 6: Choices between trisaccharides – *M. rubra*			
Melezitose	6.2713	5	0.9992
Raffinose	4.5778	5	0.997
Water	-1.482	5	0.09922
Exp. 7: Choices between most preferred mono-, di- and trisaccharides – *C. modoc*			
Fructose	3.0161	4	0.9803
Sucrose	5.1745	4	0.9967
Raffinose	4.9503	4	0.9961
Exp. 7: Choices between most preferred mono-, di- and trisaccharides – *M. rubra*			
Fructose	3.2327	5	0.9884
Sucrose	5.5709	5	0.9987
Melezitose	3.3073	5	0.9893
Water	-0.079075	5	0.47

Appendix D.

Supplementary information for Chapter 7

Supplementary tables

Table D1. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumption of aqueous amino acid solutions by colonies of *Camponotus modoc* and *Myrmica rubra.* We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMMs (CIs)	Contrast	Z ratio	P value
H1: Colonies with brood preferentially forage for EAAs rather than non-EAAs (Exps. 1–4)					
C. modoc					
1	EAAs + non-EAAs	0.77 (0.59–0.89)	EAAs + non-EAAs *vs* non-EAAs	5.09	<0.0001
	Non-EAAs	0.18 (0.08–0.35)	EAAs + non-EAAs *vs* Water	5.60	<0.0001
	Water	0.12 (0.05–0.27)	Non-EAAs *vs* Water	0.87	0.65
2	EAAs + non-EAAs	0.54 (0.46–0.62)	EAAs + non-EAAs *vs* EAAs	2.53	0.03
	EAAs	0.41 (0.32–0.50)	EAAs + non-EAAs *vs* Water	11.59	<0.0001
	Water	0.08 (0.05–0.12)	EAAs *vs* Water	8.51	<0.0001
M. rubra					
3	EAAs + non-EAAs	0.88 (0.73–0.95)	EAAs + non-EAAs *vs* non-EAAs	8.03	<0.0001
	Non-EAAs	0.07 (0.03–0.16)	EAAs + non-EAAs *vs* Water	8.78	<0.0001
	Water	0.08 (0.04–0.15)	Non-EAAs *vs* Water	-0.25	0.97
4	EAAs + non-EAAs	0.44 (0.22–0.68)	EAAs + non-EAAs *vs* EAAs	-0.16	0.97
	EAAs	0.46 (0.24–0.69)	EAAs + non-EAAs *vs* Water	2.06	0.10
	Water	0.18 (0.07–0.40)	EAAs *vs* Water	2.20	0.07

Table D2. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of the number of larvae, workers and queens in *Myrmica rubra* colonies after a 16-week rearing experiment (Exp. 5). Colonies were reared on nutrient solutions containing both essential amino acids (EAAs) and non-essential amino acids (non-EAAs; Tab. 2), or just EAAs or non-EAAs. We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Caste	Treatment	EMMs (CIs)	Contrast	Z ratio	P value
Larvae	EAAs + non-EAAs	10.9 (6.42–18.50)	EAAs + non-EAAs *vs* EAAs	4.36	<0.0001
	EAAs	2.2 (1.10–4.42)	EAAs + non-EAAs *vs* non-EAA	4.93	<0.0001
	non-EAAs	1.6 (0.75–3.44)	EAAs *vs* non-EAA	0.73	1
Workers	EAAs + non-EAAs	29.9 (28.20–31.71)	EAAs + non-EAAs *vs* EAA	2.16	0.09
	EAAs	27.7 (26.06–29.44)	EAAs + non-EAAs *vs* non-EAA	1.86	0.12
	non-EAAs	28 (26.35–29.75)	EAAs *vs* non-EAAs	-0.30	1
Queens	EAAs + non-EAAs	1.8 (1.49–2.17)	EAAs + non-EAAs *vs* EAA	-0.52	1
	EAAs	1.9 (1.61–2.24)	EAAs + non-EAAs *vs* non-EAA	-0.52	1
	non-EAAs	1.9 (1.61– 2.24)	EAA *vs* non-EAAs	0	1

Appendix E.

Supplementary information for Chapter 8

Tables and figures

Table E1. Composition of nutrient test stimuli, their ratios, and purities for chemicals used in ant foraging experiments.

Test stimuli	Chemical	Fraction of total	Supplier[a]	Purity	CAS
Sucrose	D-sucrose	1.00	SA	≥99%	57-50-1
Urea	Urea	1.00	ME	≥99%	57-13-6
Essential amino acids[b]	L-Glutamic Acid	0.091	SA	99%	56-86-0
	L-Alanine	0.091	SA	≥98%	56-41-7
	L-Isoleucine	0.091	MI	≥98%	73-32-5
	L-Leucine	0.091	SA	97%	61-90-5
	L-Valine	0.091	SA	≥98%	72-18-4
	L-Tryptophan	0.091	SA	≥98%	73-22-3
	L-Arginine	0.091	SA	≥98%	74-79-3
	L-Histidine	0.091	SA	≥99%	71-00-1
	L-Threonine	0.091	SA	≥98%	72-19-5
	L-Methionine	0.091	SA	≥98%	63-68-3
	L-Phenylalanine	0.091	SA	99%	63-91-2
Glycerides[c]	Triolein	0.125	GL	NA[h]	122-32-7
	1,2-Diolein	0.875	AK	≥97%	2442-61-7
Fatty acids[d]	Oleic acid	0.600	AA	90%	112-80-1
	Linoleic acid	0.332	CB	96%	60-33-3
	Linolenic acid	0.068	CB	70%	463-40-1
Sterols[e]	stigma sterol	0.0625	SA	~95%	83-48-7
	7-dehydrocholesterol	0.0625	SA	≥95%	434-16-2
	5α-Cholestan-3β-ol	0.0625	TRC	≥95%	80-97-7
	Cholesterol	0.8125	AK	≥99%	57-88-5
Salts[f]	$CuCl_2$	0.143	SA	97%	7447-39-4
	$FeCl_3$	0.143	OW	98%	7705-08-0
	$MnCl_2$	0.143	SA	≥99%	7773-01-5
	NaCl	0.143	FI	≥99%	7647-14-5
	$ZnCl_2$	0.143	AA	≥99%	7646-85-7
	KH_2PO_4	0.143	SA	≥99%	7778-77-0
	$MgSO_4$	0.143	CL	≥99%	7487-88-9
Vitamins[g]	p-amino benzoic acid	0.111	SA	≥99%	150-13-0
	Ascorbic acid	0.111	BS	≥97%	50-81-7
	Calcium D-pantothenate	0.111	SA	≥98%	137-08-6

	Folic acid	0.111	SA	≥97%	59-30-3
	Nicotinic acid	0.111	SA	≥98%	59-67-6
	Pyridoxin hydrochloride	0.111	SA	≥98%	58-56-0
	Riboflavin	0.111	SA	≥98%	83-88-5
	Thiamine	0.111	SA	≥99%	67-03-8
	Choline chloride	0.111	SA	≥99%	67-48-1

[a] SA = Sigma Aldrich, Burlington, MA, USA; ME = Merck, Darmstadt, Germany; MI = Millipore, Burlington, MA, USA; GL = Gries-Lab synthesis (Renyard lipid); AK = AK Scientific, Inc., Union City, CA, USA; AA = Alfa Aesar, Ward Hill, MA, USA; CB = Combi-Blocks Inc., San Diego, CA, USA; TRC = Toronto Research Chemicals Inc., Toronto, ON, Canada; OW = Oakwood Products, Inc., Estill, SC, USA; FI = Fisher Scientific International, Inc., Pittsburgh, PA, USA; CL = Caledon Laboratories Ltd., Georgetown, ON, Canada

[b] Essential amino acids listed as in Feldhaar et al., 2007.

[c] The ratio of 1,2 diolein to triolein was based on limited quantity of available triolein.

[d] Fatty acids are in ratios as tested in Hughes et al., 1994.

[e] Sterols were tested in approximate dietary ratios for insects with cholesterol being the dominant component listed in Table 3, Behmer and David Nes, 2003.

[f] We used salts listed in Feldhaar et al., 2007 but tested them in equal ratio.

[g] We used vitamins listed in Feldhaar et al., 2007 but tested them in equal ratio.

[h] The purity of triolein could not be ascertained due to its high molecular weight (885) and thus poor gas chromatography.

Table E2. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumption of aqueous solutions of sucrose (S) by colonies of *Camponotus modoc* and *Myrmica rubra*. We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMMs (95% CIs)	Pairwise	Z ratio	p-value
C. modoc					
Exp. 1	Water	0.16 (0.07–0.32)	Water *vs* 0.625% S	-0.26	0.99
	0.625% S	0.18 (0.08–0.35)	Water *vs* 1.25% S	-2.66	**0.04**
	1.25% S	0.41 (0.24–0.60)	Water *vs* 2.5% S	-1.87	0.23
	2.5% S	0.32 (0.17–0.51)	0.625% S *vs* 1.25% S	-2.41	0.08
			0.625% S *vs* 2.5% S	-1.62	0.37
			1.25% S *vs* 2.5% S	0.82	0.84
M. rubra					
Exp .2	Water	0.16 (0.07–0.32)	Water *vs* 0.625% S	-1.31	0.55
	0.625% S	0.18 (0.08–0.35)	Water *vs* 1.25% S	-2.73	**0.03**
	1.25% S	0.41 (0.24–0.61)	Water *vs* 2.5% S	-9.07	**<0.0001**
	2.5% S	0.32 (0.17–0.52	0.625% *vs* 1.25% S	-1.46	0.46
			0.625% *vs* 2.5% S	-8.35	**<0.0001**
			1.25% *vs* 2.5% S	-7.4	**<0.0001**

Table E3. Modelled estimated marginal means (EMM) and 95% confidence intervals (CI) of proportional consumption of aqueous solutions of glycerides (G), fatty acids (FA), and sterols (S) by colonies of *Camponotus modoc* and *Myrmica rubra*. We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMM (95% CI)	Pairwise	Z ratio	p-value
C. modoc – Glycerides					
3	Water	0.31 (0.16–0.52)	Water *vs* Tween + water	0.65	0.91
	Tween + Water	0.25 (0.12–0.45)	Water *vs* 0.5% G	-0.02	1.0
	0.5% G	0.32 (0.16–0.53)	Water *vs* 1.0% G	1.20	0.63
	1.0% G	0.20 (0.09–0.39)	Tween + water *vs* 0.5% G	-0.66	0.91
			Tween + water *vs* 1.0% G	0.55	0.95
			0.5% G *vs* 1.0% G	1.22	0.62
M. rubra – Glycerides					
4	Water	0.22 (0.14–0.34)	Water *vs* Tween + water	0.01	1.0
	Tween + Water	0.22 (0.14–0.34)	Water *vs* 0.5% G	-1.08	0.70
	0.5% G	0.29 (0.19–0.41)	Water *vs* 1.0% G	-0.71	0.89
	1.0% G	0.27 (0.17–0.39)	Tween + water *vs* 0.5% G	-1.09	0.69
			Tween + water *vs* 1.0% G	-0.72	0.89
			0.5% G *vs* 1.0% G	0.37	0.98
C. modoc – Fatty acids					
5	Water	0.33 (0.17–0.53)	Water *vs* Tween + water	-0.07	1.0
	Tween + Water	0.33 (0.15–0.59)	Water *vs* 1.25% FA	1.17	0.64
	1.25% FA	0.21 (0.10–0.40)	Water *vs* 2.5% FA	2.61	**0.04**
	2.5% FA	0.14 (0.09–0.22)	Tween + water *vs* 1.25% FA	1.12	0.68
			Tween + water *vs* 2.5% FA	2.31	0.09
			1.25% FA *vs* 2.5% FA	1.09	0.69
M. rubra – Fatty acids					
6	Water	0.29 (0.25–0.33)	Water *vs* Tween + water	-0.86	0.83
	Tween + Water	0.36 (0.12–0.58)	Water *vs* 1.25% FA	1.4	0.48
	1.25% FA	0.20 (0.10–0.36)	Water *vs* 2.5% FA	3.05	**0.01**
	2.5% FA	0.14 (0.07–0.25)	Tween + water *vs* 1.25% FA	1.64	0.35
			Tween + water *vs* 2.5% FA	2.66	**0.04**
			1.25% FA *vs* 2.5% FA	0.96	0.77

C. modoc – Sterols

7	Water	0.26 (0.13–0.46)	Water *vs* Tween + water	-0.02	1.0
	Tween + Water	0.27 (0.13–0.46)	Water *vs* 0.5% S	-0.49	0.96
	0.5% S	0.31 (0.16–0.52)	Water *vs* 1.0% S	1.04	0.73
	1.0% S	0.17 (0.08–0.35)	Tween + water *vs* 0.5% S	-0.47	0.97
			Tween + water *vs* 1.0% S	1.06	0.71
			0.5% S *vs* 1.0% S	1.52	0.43

M. rubra – Sterols

8	Water	0.20 (0.12–0.33)	Water *vs* Tween + water	-0.83	0.84
	Tween + Water	0.25 (0.15–0.39)	Water *vs* 0.5% S	-3.0	**0.01**
	0.5% S	0.41 (0.29–0.55)	Water *vs* 1.0% S	-0.20	1.0
	1.0% S	0.21 (0.13–0.34)	Tween + water *vs* 0.5% S	-2.21	0.12
			Tween + water *vs* 1.0% S	0.64	0.92
			0.5% S *vs* 1.0% S	2.82	**0.02**

Table E4. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumptions of aqueous solutions of salts (S) and vitamins (V) by colonies of *Camponotus modoc* and *Myrmica rubra.* We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMMs (CIs)	Pairwise	Z ratio	p-value
C. modoc - salts					
9	Water	0.27 (0.14–0.45)	Water *vs* 0.25% S	0.71	0.89
	0.25%	0.21 (0.10–0.38)	Water *vs* 0.5% S	0.02	1.0
	0.5%	0.27 (0.14–0.45)	Water *vs* 1.0% S	-0.14	1.0
	1.0%	0.28 (0.15–0.46)	0.25% S *vs* 0.5% S	-0.70	0.90
			0.25% S *vs* 1.0% S	-0.85	0.83
			0.5% S *vs* 1.0% S	-0.15	1.0
M. rubra - salts					
10	Water	0.51 (0.31–0.71)	Water *vs* 0.25% S	2.08	0.16
	0.25%	0.31 (0.21–0.43)	Water *vs* 0.5% S	3.84	**0.0007**
	0.5%	0.13 (0.05–0.27)	Water *vs* 1.0% S	7.80	**<0.0001**
	1.0%	0.03 (0.02–0.06)	0.25% S *vs* 0.5% S	2.57	0.05
			0.25% S *vs* 1.0% S	7.45	**<0.0001**
			0.5% S *vs* 1.0% S	3.12	**0.0097**
C. modoc - vitamins					
11	Water	0.25 (0.13–0.43)	Water *vs* 0.25% V	-0.24	1.0
	0.25%	0.27 (0.14–0.45)	Water *vs* 0.5% V	0.06	1.0
	0.5%	0.25 (0.13–0.42)	Water *vs* 1.0% V	-0.06	1.0
	1.0%	0.26 (0.13–0.43)	0.25% V *vs* 0.5% V	0.30	1.0
			0.25% V *vs* 1.0% V	0.19	1.0
			0.5% V *vs* 1.0% V	-0.11	1.0
M. rubra - vitamins					
12	Water	0.41 (0.28–0.56)	Water *vs* 0.25% V	3.61	**0.002**
	0.25%	0.15 (0.08–0.27)	Water *vs* 0.5% V	3.90	**0.0006**
	0.5%	0.13 (0.07–0.25)	Water *vs* 1.0% V	0.32	0.99
	1.0%	0.39 (0.25–0.54)	0.25% V *vs* 0.5% V	0.32	0.99
			0.25% V *vs* 1.0% V	-3.33	**0.005**
			0.5% V *vs* 1.0% V	-3.62	**0.002**

Table E5. **Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumptions of aqueous solutions of urea, essential amino acids (EAA) or sucrose – presented singly and in ternary combination (Blend) – by colonies of *Camponotus modoc* and *Myrmica rubra*. The concentration of each macro-nutrient type was kept the same as in the ternary blend ('unadjusted') or matched the total nutrient concentration of the blend ('adjusted'). We also report pairwise comparisons between treatments and their Tukey adjusted p-values.**

Exp. #	Treatment	EMMs (CI)	Pairwise	Z ratio	p-value
C. modoc – unadjusted					
13	Water	0.022 (0.015–0.033)	Water *vs* Urea	-7.73	**<0.0001**
	Urea	0.23 (0.13–0.40)	Water *vs* EAAs	-11.25	**<0.0001**
	EAAs	0.28 (0.19–0.39)	Water *vs* Sucrose	-3.14	**0.01**
	Sucrose	0.05 (0.03–0.08)	Water *vs* Blend	-20.36	**<0.0001**
	Blend	0.44 (0.39–0.50)	Urea *vs* EAAs	-0.63	0.97
			Urea *vs* Sucrose	5.02	**<0.0001**
			Urea *vs* Blend	-3.13	**0.01**
			EAAs *vs* Sucrose	7.22	**<0.0001**
			EAAs *vs* Blend	-3.46	**0.005**
			Sucrose *vs* Blend	-12.82	**<0.0001**
C. modoc – adjusted					
14	Water	0.03 (0.02–0.04)	Water *vs* Urea	-6.59	**<0.0001**
	Urea	0.19 (0.10–0.31)	Water *vs* EAAs	-11.34	**<0.0001**
	EAAs	0.44 (0.30–0.59)	Water *vs* Sucrose	-2.83	**0.04**
	Sucrose	0.06 (0.03–0.10)	Water *vs* Blend	-8.50	**<0.0001**
	Blend	0.32 (0.19–0.50)	Urea *vs* EAAs	-3.49	**0.004**
			Urea *vs* Sucrose	3.75	**0.002**
			Urea *vs* Blend	-1.89	0.32
			EAAs *vs* Sucrose	7.83	**<0.0001**
			EAAs *vs* Blend	1.37	0.65
			Sucrose *vs* Blend	-5.65	**<0.0001**
M. rubra – unadjusted					
15	Water	0.07 (0.04–0.12)	Water *vs* Urea	-0.41	0.99
	Urea	0.08 (0.05–0.13)	Water *vs* EAAs	-3.36	**0.007**
	EAAs	0.16 (0.11–0.22)	Water *vs* Sucrose	-7.09	**<0.0001**
	Sucrose	0.32 (0.25–0.40)	Water *vs* Blend	-8.07	**<0.0001**
	Blend	0.38 (0.30–0.45)	Urea *vs* EAAs	-2.98	**0.02**
			Urea *vs* Sucrose	-6.81	**<0.0001**

			Urea *vs* Blend	-7.82	**<0.0001**
			EAAs *vs* Sucrose	-4.31	**0.0002**
			EAAs *vs* Blend	-5.50	**<0.0001**
			Sucrose *vs* Blend	-1.29	0.70
M. rubra – adjusted					
16	Water	0.06 (0.04–0.10)	Water *vs* Urea	1.52	0.55
	Urea	0.04 (0.03–0.07)	Water *vs* EAAs	-11.73	**<0.0001**
	EAAs	0.39 (0.33–0.44)	Water *vs* Sucrose	-9.98	**<0.0001**
	Sucrose	0.31 (0.27–0.37)	Water *vs* Blend	-7.41	**<0.0001**
	Blend	0.23 (0.18–0.27)	Urea *vs* EAAs	-12.19	**<0.0001**
			Urea *vs* Sucrose	-10.64	**<0.0001**
			Urea *vs* Blend	-8.37	**<0.0001**
			EAAs *vs* Sucrose	2.47	0.09
			EAAs *vs* Blend	5.73	**<0.0001**
			Sucrose *vs* Blend	3.32	**0.008**

Table E6. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumptions of aqueous solutions of urea, essential amino acids (EAAs) or sucrose – presented in binary combinations and in a ternary blend (Blend) – by colonies of *Camponotus modoc* and *Myrmica rubra*. The concentrations and ratios of macro-nutrients in binary combinations were kept the same as in the ternary blend ('unadjusted'), or matched – at equivalent proportion – the total concentration of the ternary blend ('adjusted'). We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMMs (CIs)	Pairwise	Z ratio	p-value
C. modoc - unadjusted					
17	Water	0.03 (0.02–0.05)	Water *vs* Urea + EAAs	-8.91	**<0.0001**
	Urea + EAAs	0.33 (0.21–0.46)	Water *vs* Urea + Sucrose	-4.48	**<0.0001**
	Urea + Sucrose	0.16 (0.07–0.32)	Water *vs* EAAs + Sucrose	-6.57	**<0.0001**
	EAA + Sucrose	0.23 (0.13–0.37)	Water *vs* Blend	-11.41	**<0.0001**
	Blend	0.29 (0.25–0.34)	Urea + EAAs *vs* Urea + Sucrose	2.25	0.16
			Urea + EAAs *vs* EAAs + Sucrose	1.38	0.64
			Urea + EAAs *vs* Blend	0.62	0.97
			Urea + Sucrose *vs* EAAs + Sucrose	-1.01	0.85
			Urea + Sucrose *vs* Blend	-2.18	0.19
			EAAs + Sucrose *vs* Blend	-1.17	0.77
C. modoc - adjusted					
18	Water	0.03 (0.01–0.05)	Water *vs* Urea + EAAs	-8.33	**<0.0001**
	Urea + EAAs	0.39 (0.25–0.55)	Water *vs* Urea + Sucrose	-4.78	**<0.0001**
	Urea + Sucrose	0.15 (0.08–0.25)	Water *vs* EAAs + Sucrose	-5.90	**<0.0001**
	EAA + Sucrose	0.21 (0.12–0.35)	Water *vs* Blend	-8.41	**<0.0001**
	Blend	0.26 (0.20–0.34)	Urea + EAA *vs* Urea + Sucrose	3.52	**0.004**
			Urea + EAAs *vs* EAAs + Sucrose	2.28	0.15
			Urea + EAAs *vs* Blend	1.97	0.28
			Urea + Sucrose *vs* EAAs + Sucrose	-1.18	0.76
			Urea + Sucrose *vs* Blend	-2.42	0.11
			EAA + Sucrose *vs* Blend	-0.90	0.90
M. rubra - unadjusted					
19	Water	0.09 (0.06–0.13)	Water *vs* Urea + EAAs	-0.69	0.96
	Urea + EAAs	0.11 (0.07–0.15)	Water *vs* Urea + Sucrose	-3.77	**0.002**
	Urea + Sucrose	0.18 (0.14–0.23)	Water *vs* EAAs + Sucrose	-8.34	**<0.0001**
	EAA + Sucrose	0.33 (0.27–0.39)	Water *vs* Blend	-7.40	**<0.0001**

	Blend	0.29 (0.24–0.35)	Urea + EAAs *vs* Urea + Sucrose	-3.11	**0.02**
			Urea + EAAs *vs* EAAs + Sucrose	-7.84	**<0.0001**
			Urea + EAAs *vs* Blend	-6.85	**<0.0001**
			Urea + Sucrose *vs* EAAs + Sucrose	-5.13	**<0.0001**
			Urea + Sucrose *vs* Blend	-4.03	**0.0005**
			EAA + Sucrose *vs* Blend	1.14	0.78
M. rubra - adjusted					
20	Water	0.05 (0.04–0.07)	Water *vs* Urea + EAAs	-6.79	**<0.0001**
	Urea + EAAs	0.14 (0.12–0.17)	Water *vs* Urea + Sucrose	-6.42	**<0.0001**
	Urea + Sucrose	0.14 (0.11–0.17)	Water *vs* EAAs + Sucrose	-17.74	**<0.0001**
	EAAs + Sucrose	0.45 (0.41–0.49)	Water *vs* Blend	-11.62	**<0.0001**
	Blend	0.25 (0.22–0.29)	Urea + EAAs *vs* Urea + Sucrose	0.43	0.99
			Urea + EAAs *vs* EAA + Sucrose	-14.38	**<0.0001**
			Urea + EAAs *vs* Blend	-6.03	**<0.0001**
			Urea + Sucrose *vs* EAAs + Sucrose	-14.69	**<0.0001**
			Urea + Sucrose *vs* Blend	-6.44	**<0.0001**
			EAA + Sucrose *vs* Blend	9.14	**<0.0001**

Table E7. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumptions of aqueous solutions of essential amino acids (EAAs), sucrose (S), or both (S + EAAs) by field colonies of *Camponotus modoc* and *Myrmica rubra.* We compared proportional consumption of nutrient treatments on each of 6 dates (*C. modoc*) and 7 dates (*M. rubra*). We also report pairwise comparisons between treatments within a given date and their Tukey adjusted p-values. For each date, different letters in parentheses behind treatments indicate statistically significant differences in consumptions of nutrient solutions.

Exp. #	2021	Treatment	EMMs (CIs)	Pairwise	Z ratio	p-value
C. modoc						
21	18 June	Water (b)	0.12 (0.05–0.24)	Water *vs* EAAs	-4.25	**0.0001**
		EAAs (a)	0.43 (0.31–0.55)	Water *vs* Sucrose	0.58	0.94
		Sucrose (b)	0.10 (0.05–0.17)	Water *vs* S + EAAs	-3.59	**0.002**
		S + EAAs (a)	0.37 (0.25–0.50)	EAA *vs* Sucrose	5.94	**<0.0001**
				EAA *vs* S + EAAs	0.81	0.85
				Sucrose *vs* S + EAAs	-5.08	**<0.0001**
	03 July	Water (c)	0.05 (0.03–0.10)	Water *vs* EAAs	-7.25	**<0.0001**
		EAAs (a)	0.42 (0.30–0.55)	Water *vs* Sucrose	-2.92	**0.02**
		Sucrose (b)	0.16 (0.08–0.28)	Water *vs* S + EAAs	-7.20	**<0.0001**
		S + EAAs (a)	0.38 (0.28–0.49)	EAA *vs* Sucrose	3.65	**0.002**
				EAA *vs* S + EAAs	0.57	0.94
				Sucrose *vs* S + EAAs	-3.40	**0.004**
	23 July	Water (b)	0.09 (0.05–0.15)	Water *vs* EAAs	-5.15	**<0.0001**
		EAAs (a)	0.41 (0.25–0.58)	Water *vs* Sucrose	-0.61	0.93
		Sucrose (b)	0.11 (0.06–0.20)	Water *vs* S + EAAs	-5.84	**<0.0001**
		S + EAAs (a)	0.35 (0.26–0.45)	EAA *vs* Sucrose	4.19	**0.0002**
				EAA *vs* S + EAAs	0.72	0.89
				Sucrose *vs* S + EAAs	-4.44	**0.0001**
	09 Aug.	Water (c)	0.012 (0.092–0.016)	Water *vs* EAAs	-21.54	**<0.0001**
		EAAs (a)	0.58 (0.46–0.69)	Water *vs* Sucrose	-1.78	0.28
		Sucrose (c)	0.016 (0.012–0.021)	Water *vs* S + EAAs	-15.95	**<0.0001**
		S + EAAs (b)	0.39 (0.27–0.52)	EAA *vs* Sucrose	20.26	**<0.0001**
				EAA *vs* S + EAAs	2.65	**0.04**
				Sucrose *vs* S + EAAs	-14.81	**<0.0001**
	24 Aug.	Water (b)	0.05 (0.03–0.09)	Water *vs* EAAs	-9.31	**<0.0001**
		EAAs (a)	0.44 (0.35–0.55)	Water *vs* Sucrose	-1.01	0.75
		Sucrose (b)	0.08 (0.04–0.14)	Water *vs* S + EAAs	-9.72	**<0.0001**
		S + EAAs (a)	0.43 (0.35–0.51)	EAA *vs* Sucrose	7.26	**<0.0001**
				EAA *vs* S + EAAs	0.32	0.99
				Sucrose *vs* S + EAAs	-7.45	**<0.0001**

	07 Sept.	Water (c)	0.04 (0.03–0.06)	Water *vs* EAAs	-14.76	**<0.0001**
		EAAs (a)	0.60 (0.48–0.70)	Water *vs* Sucrose	-0.60	0.93
		Sucrose (c)	0.05 (0.03–0.07)	Water *vs* S + EAAs	-7.08	**<0.0001**
		S + EAAs (b)	0.30 (0.17–0.47)	EAA *vs* Sucrose	13.65	**<0.0001**
				EAA *vs* S + EAAs	3.62	**0.002**
				Sucrose *vs* S + EAAs	-6.52	**<0.0001**
M. rubra						
22	21 May	Water (c)	0.004 (0.002–0.007)	Water *vs* EAAs	-12.98	**<0.0001**
		EAAs (b)	0.10 (0.07–0.14)	Water *vs* Sucrose	-20.75	**<0.0001**
		Sucrose (a)	0.45 (0.36–0.54)	Water *vs* S + EAAs	-22.34	**<0.0001**
		S + EAAs (a)	0.45 (0.38–0.52)	EAA *vs* Sucrose	-9.46	**<0.0001**
				EAA *vs* S + EAAs	-10.62	**<0.0001**
				Sucrose *vs* S + EAAs	-0.08	1.0.
	17 June	Water (d)	0.003 (0.002–0.004)	Water *vs* EAAs	-20.54	**<0.0001**
		EAA (c)	0.19 (0.15–0.25)	Water *vs* Sucrose	-24.57	**<0.0001**
		Sucrose (b)	0.36 (0.29–0.42)	Water *vs* S + EAAs	-28.89	**<0.0001**
		S + EAAs (a)	0.45 (0.40–0.50)	EAA *vs* Sucrose	-4.87	**<0.0001**
				EAA *vs* S + EAAs	-8.45	**<0.0001**
				Sucrose *vs* S + EAAs	-2.83	**0.02**
	04 July	Water (c)	0.03 (0.02–0.04)	Water *vs* EAAs	-10.63	**<0.0001**
		EAAs (b)	0.20 (0.15–0.26)	Water *vs* Sucrose	-16.13	**<0.0001**
		Sucrose (a)	0.40 (0.34–0.47)	Water *vs* S + EAAs	-13.08	**<0.0001**
		S + EAAs (a)	0.37 (0.28–0.48)	EAA *vs* Sucrose	-5.67	**<0.0001**
				EAA *vs* S + EAAs	-4.02	**0.0003**
				Sucrose *vs* S + EAAs	0.52	0.95
	25 July	Water (b)	0.23 (0.20–0.26)	Water *vs* EAAs	-0.30	0.99
		EAAs (b)	0.24 (0.21–0.26)	Water *vs* Sucrose	-0.98	0.76
		Sucrose (b)	0.25 (0.23–0.26)	Water *vs* S + EAAs	-3.83	**0.0007**
		S + EAAs (a)	0.29 (0.27–0.31)	EAA *vs* Sucrose	-0.79	0.86
				EAA *vs* S + EAAs	-4.27	**0.0001**
				Sucrose *vs* S + EAAs	-3.98	**0.0004**
	11 Aug.	Water (c)	0.01 (0.007–0.016)	Water *vs* EAAs	-14.13	**<0.0001**
		EAAs (b)	0.26 (0.18–0.36)	Water *vs* Sucrose	-15.57	**<0.0001**
		Sucrose (ba)	0.31 (0.23–0.41)	Water *vs* S + EAAs	-20.65	**<0.0001**
		S + EAAs (a)	0.42 (0.35–0.49)	EAA *vs* Sucrose	-0.89	0.81
				EAA *vs* S + EAAs	-3.18	**0.008**
				Sucrose *vs* S + EAAs	-2.25	0.11
	01 Sept.	Water (d)	0.003 (0.002–0.005)	Water *vs* EAAs	-19.03	**<0.0001**
		EAAs (c)	0.19 (0.16–0.24)	Water *vs* Sucrose	-19.85	**<0.0001**
		Sucrose (b)	0.32 (0.25–0.40)	Water *vs* S + EAAs	-24.08	**<0.0001**
		S + EAAs (a)	0.48 (0.42–0.55)	EAA *vs* Sucrose	-3.61	**0.002**

			EAA *vs* S + EAAs	-9.00	**<0.0001**
			Sucrose *vs* S + EAAs	-3.91	**0.0005**
13 Sept.	Water (c)	0.003 (0.002–0.006)	Water *vs* EAAs	-10.76	**<0.0001**
	EAAs (b)	0.16 (0.08–0.28)	Water *vs* Sucrose	-11.22	**<0.0001**
	Sucrose (b)	0.22 (0.11–0.39)	Water *vs* S + EAAs	-19.58	**<0.0001**
	S + EAAs (a)	0.60 (0.47–0.71)	EAA *vs* Sucrose	-0.96	0.77
			EAA *vs* S + EAAs	-5.80	**<0.0001**
			Sucrose *vs* S + EAAs	-4.34	**0.0001**

Table E8. Modelled estimated marginal means (EMMs) and 95% confidence intervals (CIs) of proportional consumptions of aqueous solutions of essential amino acids (EAA), sucrose (S), or both (S + EAAs) by field colonies of *Lasius niger* and *Formica aserva*. We also report pairwise comparisons between treatments and their Tukey adjusted p-values.

Exp. #	Treatment	EMMs (CIs)	Pairwise	Z ratio	p-value
L. niger					
23	Water	0.07 (0.04–0.12)	Water *vs* EAAs	-7.07	**<0.0001**
	EAAs	0.32 (0.25–0.39)	Water *vs* Sucrose	-6.08	**<0.0001**
	Sucrose	0.27 (0.21–0.34)	Water *vs* S + EAAs	-7.39	**<0.0001**
	S + EAAs	0.34 (0.27–0.41)	EAAs *vs* Sucrose	1.24	0.60
			EAAs *vs* S + EAAs	-0.42	0.98
			Sucrose *vs* S + EAAs	-1.65	0.35
F. aserva					
24	Water	0.18 (0.10–0.30)	Water *vs* EAAs	-3.28	**0.006**
	EAAs	0.40 (0.28–0.54)	Water *vs* Sucrose	-0.11	1.0
	Sucrose	0.18 (0.10–0.30)	Water *vs* S + EAAs	-1.65	0.35
	S + EAAs	0.28 (0.18–0.41)	EAAs *vs* Sucrose	3.17	**0.008**
			EAAs *vs* S + EAAs	1.69	0.33
			Sucrose *vs* S + EAAs	-1.54	0.41