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

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ABSTRACT

Trail pheromones of ants guide nest mates to a food source. Applications of synthetic trail pheromone could guide ants to poisoned food baits, which may expedite the demise of nests and help control invasive ant species. The trail pheromone of the invasive European fire ant (EFA), *Myrmica rubra* Linnaeus (Hymenoptera: Formicidae), has previously been identified as 3-ethyl-2,5-dimethylpyrazine. To facilitate its development as an operational EFA control tactic, our objectives were to determine the effects of (1) pheromone purity (isometrically pure or isomeric mixture), (2) pheromone dose [2, 20, 200, 2,000 ant equivalents (AEs)], and (3) type of pheromone placement (pheromone encircling a food source rather than leading towards it) on ant recruitment to baits. In laboratory binary choice experiments, isomerically pure and impure trail pheromone prompted similar recruitment responses of ants. The presence of pheromone, irrespective of dose, enhanced the recruitment of ants to food baits, with the dose of 200 AEs eliciting the strongest recruitment responses (2 AEs: 61% of foraging ants; 20 AEs: 57%; 200 AEs: 69%; 2000 AEs: 59%). Pheromone applied in a line leading towards the food bait, but not in a circle surrounding a food bait, was effective in recruiting ants, suggesting that 3-ethyl-2,5-dimethylpyrazine has a guiding but not an attractive function to EFAs.

INTRODUCTION

Many ant species use a trail pheromone to coordinate foraging efforts (Billen and Morgan 1998). When a foraging ant finds a food source, she returns to the nest depositing trail pheromone along the route. Nest mates then use this pheromone trail to find their way to the food source, reinforcing the trail in the process. Foragers of some ant species may deposit more pheromone, and thus recruit more nest mates, when they have found a high-quality food source (de Biseau et al. 1991; Czaczkes et al. 2015). Essentially, nest mates make collective decisions about the food sources they want to exploit. Trail pheromone–guided foraging is common in ants (Czacskes et al. 2015), including the European fire ant (EFA), *Myrmica rubra* Linnaeus (Hymenoptera: Formicidae) (Cammaerts-Tricot 1973).

In their native range in Europe, EFAs can live in single colonies with hundreds of workers and several queens (Fokuhl et al. 2007) but often form multi-nest colonies and super-colonies (large networks of interconnected nests) that eradicate rivalling ant species (Huszár et al. 2014). European fire ants prey on small invertebrates and tend to aphids, from which they collect honeydew. They also collect elaiosomes and disperse seeds (Fokuhl et al. 2007). In their invaded range (the east and west coasts of North America), the ants do not engage in nuptial flights (Groden and Drummond 2005) where winged queens mate with males and then land elsewhere to start a new nest. Instead, new nests occur by "budding" (queens and workers leaving the original nest and establishing a new nest nearby) without nuptial flights. As a result, local population densities of EFAs can become so high that they exterminate native ant species (Naumann and Higgins 2015). Because the species swarms and stings aggressively when disturbed, it can render gardens, lawns and parks unusable (Garnas 2004; Saltman 2016).

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European fire ants are efficient foragers, apparently locating resources faster than other ant species (Holway 1999; Groden et al., unpubl. data, as cited in Garnas 2004). To coordinate foraging efforts, the ants use a trail pheromone (3-ethyl-2,5-dimethylpyrazine) that they release from the poison gland (Evershed et al. 1982). Individual ants follow trails of synthetic pheromone (Evershed et al. 1981), but it is not yet known whether synthetic pheromone can be exploited for EFA control.

Current EFA control methods are not very effective. Excavating nests and spraying them with permethrins is labour intensive and may need to be repeated if one or more queens have been missed in the process (Higgins 2017). Topical insecticide treatments of nests are ineffective, because they kill only those ants that happen to be above ground at the time of treatment. The concept of lethal food baits to control EFAs is appealing, because they forage socially and through trophallaxis might effectively distribute any lethal agent among nest mates (Brian and Abbott 1977). The efficacy of lethal food baits likely hinges upon attractive food odorants and/or synthetic trail pheromone to guide foraging ants to food baits.

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

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

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

A lethal food source must be deployed together with synthetic trail pheromone to achieve the demise of fire ant nests. The placement method of synthetic trail pheromone likely determines its effectiveness for recruitment of ants to lethal food baits. Ants deposit trail pheromone in a line to guide nest mates towards a food source (Cammaerts-Tricot 1978), but for ant control it would be most efficient to apply pheromone directly to the food bait rather than laying down a pheromone trail towards it. By simply adding trail pheromone directly to lethal baits, bait consumption by Argentine ants, *Linepithema humile* Mayr (Hymenoptera: Formicidae), increased and thus resulted in greater ant mortality and lower ant activity in the field (Greenberg and Klotz 2000; Welzel and Choe 2016). However, the recruitment effect of this type of pheromone placement may depend on both the volatility of the pheromone and the propensity of foraging ants to be attracted to, rather than guided by, trail pheromones.

Our overall objective was to determine whether the synthetic trail pheromone of EFAs (3-ethyl-2,5-dimethylpyrazine) can be deployed to increase recruitment of nest mates to food baits. Our specific objectives were to determine the effects of (1) pheromone purity (isometrically pure or isomeric mixture), (2) pheromone dose (2–2,000

ant equivalents), and (3) type of pheromone placement (pheromone encircling a food bait rather than leading towards it) on ant recruitment to baits.

MATERIALS AND METHODS

Colony collections. We collected EFA colonies in the spring and summer of 2016–2018 from Inter River Park (North Vancouver, British Columbia), the Burnaby and Region Allotment Garden (Burnaby, British Columbia), and the VanDusen Botanical Garden (Vancouver, British Columbia). To locate nests, we walked in a transect while disturbing the soil by shuffling our feet. Because the ants respond quickly when disturbed, it is easy to locate a nest entrance. Wearing nitrile gloves to protect ourselves from stings, we excavated about 30 cm^3 of soil surrounding a nest entrance, and placed it in a large bucket (19 L, 38 cm tall × 30 cm diam.). We slowly sifted through this soil by hand, collecting about 10 queens, 200–300 workers, and 50–100 larvae and pupae from each nest. We transferred the ants to artificial nest housings (see below), producing and maintaining a total of 41 colonies for laboratory bioassays.

Rearing of experimental ants. We kept ant colonies indoors in the Science Research Annex (49° 16'33" N, 122° 54'55" W) of Simon Fraser University at a temperature of 25 °C and a photoperiod of 12L:12D. The rearing protocol took into account that colonies need both an enclosed nest housing (hereinafter referred to as the "nest-box") and a surrounding foraging arena to exhibit normal behavior (Drees and Ellison 2002; Fig. 1A). Each nest-box consisted of a small plastic container (15 × 15 × 9 cm), two-thirds of which was filled with potting soil (Sunshine® Mix #4, Sungro, Agawam, Massachusetts, United States). A 10-cm^2 hole in nest-box lids was covered with plastic mesh (Lumite Saran fabric, 10 ml) to allow for ventilation and water misting (see below). Each nest-box was placed inside a foraging arena, consisting of a plastic tote (41 × 29 × 24 cm or 58 × 43 × 31 cm) fitted with a mesh-covered hole (10 × 10 cm) in the lid to allow air flow. Ants entered and exited the nest-box through a 15-cm-long Nalgene tubing (3.175 mm diam.; Nalgene 180 PVC non-toxic autoclavable Lab/FDA/USB V1 grade; Thermo Scientific, Waltham, Massachusetts, United States). The 5-cm-wide, upper-most rim of each foraging arena was coated with a 1:1 mixture of paraffin oil [White, Anachemia, Lachine (Montreal), Quebec, Canada] and petroleum jelly (Vaseline) to prevent ants from escaping.

Twice per week, we added one source of protein (canned tuna, cat food, dog food, tofu, canned beans, canned chicken, dehydrated shrimp, anchovy paste, dead mealworms, dead blow flies, dead crickets, sunflower seeds, pumpkin seeds, luncheon meat, or corned beef), and one source of carbohydrates (canned oranges, apple slices, apple sauce, grapes, candy, honey, sugar water, cranberry sauce, or raisins) to the foraging arena, thus allowing ants to leave their nest-box and forage (Fig. 1A). We provided the large variety of foods to minimize the possibility that ants "learned" to forage for a specific food type. We provided water in a test tube fitted with a piece of cotton, which we replaced whenever it became moldy or dry. Twice per week, we rehydrated nest-boxes by spraying water through the mesh window.

General design of the binary choice biossary. We deprived colonies of food (but not water) 5–7 days prior to bioassays, testing each colony (n = 14–20 depending on the experiment; see Table 1) only once for each stimulus and separating bioassays by at least 24 h. We ran experimental replicates during the ants' photoperiod in six circular arenas (122 cm diam. × 40 cm height) housed in a dedicated bioassay room at 22–23 ºC (Fig. 1B). To initiate a bioassay, we temporarily closed the entrance tube to a nest-box with a piece of cotton. We then removed this nest-box from the foraging arena (Fig. 1A) and placed it in a circular arena, such that the nest entrance tube was perpendicular to two strips of filter paper (each 30 × 3 cm) taped to the arena floor. To provide a food source for foraging ants, we placed a mixture of macerated apples and mealworms (1:1 ratio; 2 g total) on top of a circular piece of damp cotton (9 cm diam.) at the distal end of each

strip. After the ants (in their transferred nest-box) had acclimatized in the bioassay arena for 10 min., we treated—by random assignment—one of the two paper strips with a 25-µl aliquot of synthetic trail pheromone dissolved in pentane and the other strip with a pentane control. Immediately following the application of test stimuli, we opened the nest-box entrance to initiate the bioassay. We terminated all experimental replicates after 2 h (when ant foraging activity peaked according to preliminary testing), at which time we counted the number of ants present on food baits or cotton circles, using these data as the response variable for statistical analyses. We collected all foraging ants by aspirator or hand and returned them together with the nest-box to their original foraging arena. We tested pheromone in ant equivalents (AEs), with a mean pheromone amount of 5.8 ng occurring in a single worker ant (Cammaerts et al. 1981).

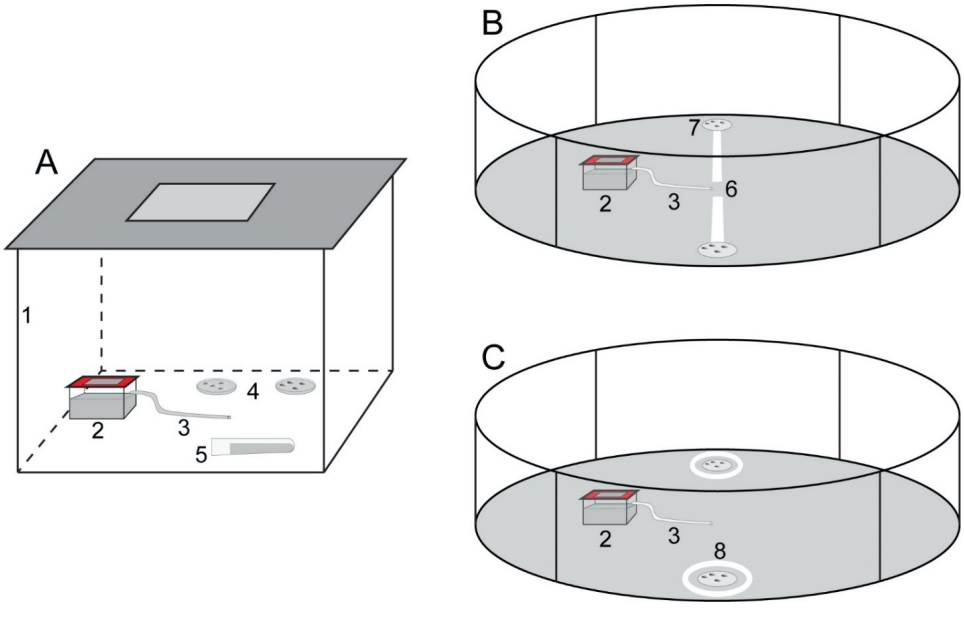

Figure 1. Illustrations of the various nest-box components and design for the study. **A.** The set-up for maintaining ant colonies in the insectary annex consisted of a foraging arena (58 × 43 × 31 cm) (1), which housed the ants' NEST-BOX (15 × 15 × 9 cm) (2) fitted with a Nalgene tubing (3.1 mm diam, 15 cm long) (3) for nest entry and exit, and was provisioned with sources of food and water presented in Petri dishes (4) and in form of a moist cotton plug confining a water reservoir inside a test tube (5). **B** and **C**. The experimental designs for testing the effect of synthetic trail pheromone on foraging decisions by ants. For each replicate in Design B, the nest-box (2) was placed inside a large circular bioassay arena such that the entry, and exit tubing (3) was perpendicular to two filter paper strips (30 × 3 cm) (6), each leading to a circular piece of damp cotton (7 cm diam.) with a food bait (7). For each replicate in Design C, each deposit of food bait was surrounded by a circular filter paper strip (15 cm diam., 2 cm wide) (8), one of which was treated with synthetic trail pheromone and the other with a solvent control.

Table 1. List of research objectives (O) and stimuli tested in Experiments 1–3.

Experiment #	Test stimuli (T)	Replicates[e]
	O_1: Determine the effect of pheromone isomer	
1	T_1: pure pheromone[a]; T_2: pheromone mixture[b] (200 AEs[c] tested for both T_1 and T_2)	14
	O_2: Determine the optimal dose of pheromone[d]	
2	T_1: 2 AEs; T_2: Solvent control	20
	T_1: 20 AEs; T_2: Solvent control	20
	T_1: 200 AEs; T_2: Solvent control	20
	T_1: 2,000 AEs; T_2: Solvent control	15
	O_3: Determine the effect of pheromone[d] placement	
3	T_1: Pheromone circle around bait (200 AEs); T_2: Solvent control circle around bait	20

[a]3-ethyl-2,5-dimethylpyrazine;
[b]mixture of 3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine at a 1:1 ratio;
[c]AE = ant equivalent of trail pheromone (5 ng)
[d]tested as pheromone mixture (see b)
[e]Replicates equal the number of ant colonies tested

SPECIFIC EXPERIMENTS

Experiment 1. Effect of 3-ethyl-2,5-dimethylpyrazine alone and in combination with isomeric 3-ethyl-2,6-dimethylpyrazine on trail-following responses of ants. The commercial source of the EFA trail pheromone component 3-ethyl-2,5-dimethylpyrazine (Acros Organics, part of Thermo Fisher Scientific, New Jersey, United States) is an isomeric mixture of 3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine at a 1:1 ratio. To isolate the natural (EFA-produced) isomer from the commercial isomeric mixture, we employed a high-performance liquid chromatograph (HPLC) (Waters Corporation, Milford, Massachusets, United States) fitted with a Synergy Hydro Reverse Phase C18 column (250 mm × 4.6 mm, 4 µ; Phenomenex, Torrance, California, United States) and operated by a HPLC System (600 Controller, 2487 Dual Absorbance Detector, Delta 600 Pump). Eluting the isomeric mixture with a 0.75-$mL^{-1\ min}$ flow of acetonitrile separated the two isomers but without baseline resolution. By collecting only the second half of the later eluting target isomer (3-ethyl-2,5-dimethylpyrazine) peak, we could obtain material for bioassays with 83% to 93% purity.

To determine whether the non-natural isomer (3-ethyl-2,6-dimethylpyrazine) in the isomeric mixture had any adverse effect on trail-following responses of EFAs, we used the general two-choice bioassay design described above (Fig. 1B), and tested the isolated synthetic trail pheromone component 3-ethyl-2,5-dimethylpyrazine alone [200 AEs (1,000 ng per trail)] *versus* the isomeric mixture containing 3-ethyl-2,5-dimethylpyrazine at the same amount.

Experiment 2. Effect of trail pheromone dose on trail-following responses of ants. To determine the trail pheromone dose that elicits the strongest trail-following responses by EFAs, we tested each of four doses of synthetic trail pheromone (10, 100, 1,000, 10,000 ng, equivalent to 2, 20, 200 and 2,000 AEs, respectively) dissolved in pentane (25 µL) *versus* a pentane control (25 µL) (Table 1). We applied the pheromone treatment stimulus and the solvent control stimulus in 30-cm-long streaks on two non-

overlapping paper strips (each 30 × 3 cm) secured in a straight line to the bioassay arena floor (see general experimental design; Fig. 1B).

Experiment 3. Effect of trail pheromone encircling a food bait on ant recruitment. To determine whether EFAs respond to trail pheromone encircling a food source (Fig. 1C) rather than leading towards it (Fig. 1B), we modified the experimental design of Experiments 1 and 2. We surrounded each of the two food baits with a circular strip of filter paper (15 cm diam.; 2 cm wide; cut from a circular filter paper; Fig. 1C) and treated one strip with synthetic trail pheromone (isomer mixture, 200 ng) and the other with a solvent control. This revised binary choice experimental design took into account that the effects of trail pheromone encircling a food source or leading towards it could not be compared directly because the linear trail would start near the nest entrance and thus immediately bias the recruitment response of ants.

STATISTICAL ANALYSIS

We analyzed the data of Experiment 1 (effect of pure and isomeric pheromone on trail-following responses of ants) and of Experiment 3 (effect of trail pheromone placement on trail-following responses of ants) in JMP (SAS Institute Inc., Cary, North Carolina, United States) with a χ^2-test against a theoretical 50:50 distribution. We analyzed the data of Experiment 2 (effect of trail pheromone dose (tested as ant equivalents) on trail-following responses of ants] using a general linear mixed model with a binomial distribution and a logit link function, using the GLIMMIX procedure in SAS. Ant equivalents were a fixed effect, and nest origin was a random effect. The over-dispersion in the model was accounted for by scaling the standard errors proportional to the deviance. We ran a Tukey-Kramer multiple comparisons test for pairwise comparisons between treatment groups.

RESULTS

Experiment 1: Effect of 3-ethyl-2,5-dimethylpyrazine alone and in combination with isomeric 3-ethyl-2,6=dimethylpyrazine on trial following responses of ants. There was no difference in the proportion of ants that were recruited to a food bait by the trail pheromone 3-ethyl-2,5-dimethylpyrazine alone or in combination with isomeric 3-ethyl-2,6-dimethylpyrazine (χ^2 = 0.016, n = 13, df = 12, p = 0.898; mean number of responding ants ± SE (mra ± SE): 61.2 ± 17.3; Fig. 2).

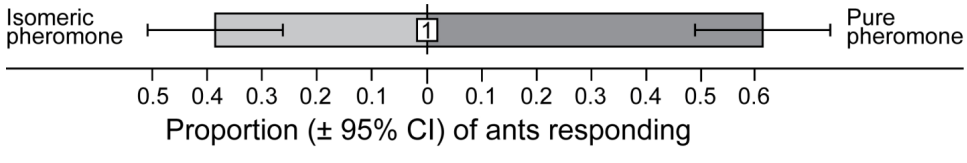

Figure 2. Mean proportion of European fire ants present in binary choice arena bioassays (see Fig. 1B) on cotton pads with food bait in response to pure pheromone (3-ethyl-2,5-dimethylpyrazine) or isomeric pheromone (3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine) applied to the paper strip leading to the food bait; χ^2-test, $p > 0.05$. The number in the bar centre (1) represents the one replicate where the nest was not responding (total number of replicates run: n = 14; mean number of responding ants ± SE: 61.23 ± 17.34).

Experiment 2: Effect of trail pheromone dose on trail-following responses of ants (Exp. 2). The presence of trail pheromone did affect the recruitment response of ants ($F_{3,65} = 11.15$, $p < 0.0001$). When trail pheromone was tested at 2 AEs, it recruited 61% of the foraging ants to the corresponding food bait (n = 20, t = 3.27, p = 0.002; mra ± SE: 35.0 ± 7.5; Fig. 3). Trail pheromone tested at 20 and 200 AEs recruited 57% and 69% of foraging ants, respectively (20 AEs: n = 19, t = 2.09, p = 0.04; mra ± SE: 36.2 ± 10.1; 200 AEs: n = 20, t = 5.99, p < 0.001; mra ± SE: 63.4 ± 12.4). At the high dose of 2,000 AEs, the effect decreased to 59% of foraging ants (n = 11, t = 2.27, p = 0.03; mra ± SE: 49.8 ± 8.7). The recruitment effect of the 200-AE dose exceeded that of the other pheromone doses tested (200 *vs* 2: t = 0.002, p = 0.01; 200 *vs* 20: t = -5.06, p < 0.0001; 200 *vs* 2,000: t = 4.34, p = 0.0003; Tukey-Kramer analyses).

Experiment 3: Effect of trail pheromone placement on ant recruitment to bait. When trail pheromone was applied at the most effective dose (200 AEs) on a filter paper strip encircling the food bait (Fig. 1C) instead of leading towards it (Fig. 1B), the pheromone failed to recruit ants to the food bait ($\chi^2 = 0.003$, n = 19, df = 18, p = 0.958; mra ± SE: 36.5 ± 7.9; Fig. 4), suggesting that the ants did not sense the pheromone.

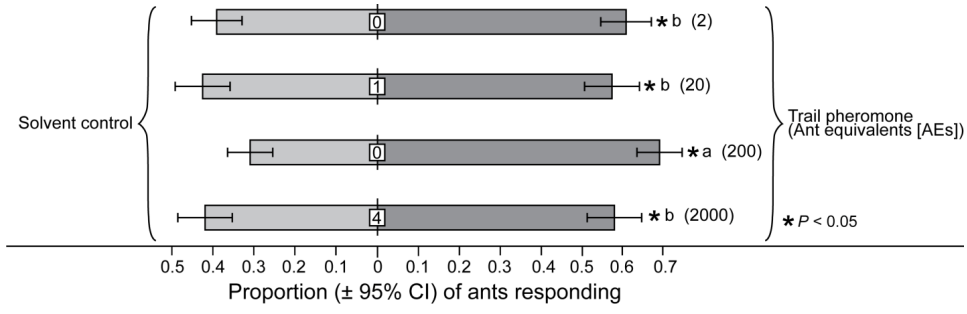

Figure 3. Mean proportion of European fire ants present in binary choice arena bioassays (Fig. 1B) on cotton pads with food bait in response to isomeric pheromone (3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine) applied at 2, 20, 200 or 2,000 ant equivalents (AEs; 1 AE = 5 ng of 3-ethyl-2,5-dimethylpyrazine) to the paper strip leading to the food bait, each pheromone dose tested *versus* a solvent control. An asterisk (*) indicates a significant preference for the pheromone stimulus. General linear mixed model, $p < 0.05$; the 200 AE trail pheromone dose was more effective than all others in recruiting ants to the food bait (Tukey-Kramer test adjusted for multiple comparisons, $p < 0.05$). The numbers in bar centres represent the number of replicates where the nest was not responding (total number of replicates run: n = 20 for each of 2, 20 and 200 AEs; n = 15 for 2,000 AEs; mean number of responding ants ± SE: 2 AEs: 34.95 ± 7.45; 20 AEs: 36.21 ± 10.18; 200 AEs: 63.40 ± 12. 43; 2,000 AEs: 49.81 ± 8.72).

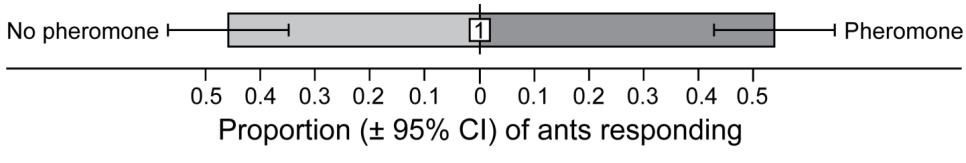

Figure 4. Mean proportion of European fire ants present in binary choice arena bioassays on food baits surrounded by a circular filter paper strip that was either treated or not treated (control) with synthetic trail pheromone (1,000 ng of a synthetic mixture of 3-ethyl-2,5-dimethylpyrazine and 3-ethyl-2,6-dimethylpyrazine) (Fig. 1C). The trail pheromone near the food bait had no effect on recruitment responses of ants; (χ^2-test, $p > 0.05$. The number in the bar centre (1) represents the one replicate where the nest was not responding (total number of replicates run: n = 20; mean number of responding ants ± SE: 36.47 ± 7.91).

DISCUSSION

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

Affordability of pheromone-based control tactics is a key factor in their development and sustained use. Pheromone-based pest control tactics are typically species-specific, causing fewer non-target effects. This, in turn, makes pheromone-based control tactics more expensive than conventional insecticides that control a wide range of pest insects. Low pheromone synthesis costs contribute to the affordability of pheromone-based control tactics and can sometimes be achieved by producing a mixture of optical or structural isomers rather than stereo-specifically pure pheromone. The commercially available and relatively affordable source of the EFA trail pheromone contains not only the trail pheromone 3-ethyl-2,5-dimethypyrazine but also 3-ethyl-2,6-dimethylpyrazine as a non-pheromonal structural isomer. The presence of optical or structural isomers in pheromone lures is known to sometimes interfere with the optimal effectiveness of the pheromone. For example, the attractiveness of synthetic (+)-disparlure, the sex pheromone of the gypsy moth, *Lymantria dispar* Linnaeus (Lepidoptera: Erebidae) (Bierl et al. 1970), is reduced in the presence of its antipode (-)-disparlure in a racemic pheromone lure (Miller et al. 1977). Similarly, tetradecenyl acetates with a double bond near C11 added to the sex pheromone (*Z*)-11-tetradecenyl acetate of the red-banded leaf roller, *Argyrotaenia velutinana* (Walker) (Lepidoptera: Tortricidae), greatly decreases pheromonal attraction of male moths (Roelofs and Comeau 1971). In light of these findings, it was important to determine whether a non-pheromonal isomer impurity (3-ethyl-2,6-dimethylpyrazine) in the commercial source of the EFA trail pheromone had adverse effect on trail-following responses of EFAs. As both pure and isomerically impure synthetic trail pheromone prompted similar trail-following responses by EFAs (Fig. 2), use of isomerically impure pheromone can now be considered for operational development.

The amount of trail pheromone deposited by ants or applied experimentally affects the trail-following response of nest mates, as shown in carpenter ants, *Camponotus* spp. (Kohl et al. 2001, 2003; Renyard et al. 2019), the leaf-cutting ant *Atta sexdens sexdens* (Morgan et al. 2006), and the EFA (Evershed et al. 1982). Also in our study with the EFA, trail-following responses were pheromone dose-dependent. As little as 2 AEs of trail pheromone (0.33 ng/cm) were sufficient to enhance recruitment of EFAs to food baits (Fig. 3), but a dose of 200 AEs (33 ng/cm) was more effective, recruiting on average 12% more foraging ants. The effect was still present, but decreased, with the highest dose (2,000 AEs or 330 ng/cm) (Fig. 3). These data differ from a previous report (Evershed et al. 1982) that a trail pheromone dose of only 0.0319 ng/cm triggered the strongest trail-following responses. These differences are not surprising given that pheromone behavior in ants is very context-dependent (Vander Meer and Alonso 1998). Evershed et al. (1982) presented a circular trail to groups of 25 or 50 EFA workers in the absence of a food bait, recording the ants' responses for 15 min. We, in contrast, offered an entire nest [at least 100 EFA workers per nest; 15–20 nests (see Table 1)] a choice between two paper strips treated with either a solvent control or the EFA trail pheromone, each strip leading to a food bait where we counted the number of recruited ants 2 h after bioassay initiation. The decreased activity of the highest trail pheromone dose (2,000 AEs) may reflect the behavioural choices of ants to ignore seemingly overcrowded trails that do not allow for efficient foraging (Dussutour et al. 2004). It also may be a result of

sensory overload, an effect that has been used to disrupt foraging behavior in the Argentine ant (Suckling et al. 2011; Sunamura et al. 2011).

Trail pheromones of ants may embody some (Möglich et al. 1974) or all (Vander Meer et al. 1990) of the following functions: orientation induction (prompting trail following by nest-mates), orientation (guiding foragers along trails), and short-range attraction (attracting foragers to trails). These functions may be mediated by a single-component pheromone or a multiple-component pheromone blend (Jackson et al. 1990). To test whether the single-component trail pheromone of EFAs has not only a guiding function but also an attractive function, we deployed either the trail pheromone or a solvent control on a circular paper strip surrounding a food bait, each bait placed 30 cm away from the nest entrance. The very similar numbers of ants recruited to these two food baits (Fig. 4) provide evidence that the EFA trail pheromone does not function as an attractant, despite its volatility. Keeping a low profile by using a trail pheromone without attractive function may be advantageous in settings of high nest density, where ants could otherwise readily eavesdrop on their neighbors' pheromone trails and exploit them (Chalissery et al. 2019)

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

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

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