Communication disruption of red imported fire ants (Hymenoptera: Formicidae) and reduced foraging success

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Abstract

Invasive pest ants often coordinate resource retrieval and colony expansion through the use of recruitment pheromones to optimise their foraging; the potential for disruption of trail pheromone communication deserves investigation as a new biologically-based method to control pest ant species. Workers from monogyne red imported fire ant colonies following trails to feed on crickets were presented greater than normal quantities of their orientation trail pheromone, Z,E-α-farnesene. The pheromone was presented as either a 300 µg point source or a linear source at 5.5 µg / cm. Ant behaviour was video recorded from above and digitised. Ants following a 10 ng / cm synthetic trail of Z,E-α-farnesene past an upwind source of the same synthetic trail pheromone took 2.4-fold (single release points) or three-fold (linear source) longer to discover the bait and remove it than ants following trails in the absence of pheromone treatments. About half (48%) of the fire ants that went past the linear sources of trail pheromone were prevented from arrival at the food compared with untreated controls (100% arrival), and ant trails had significantly lower trail integrity, measured as r^2 (obtained by linear regression), both towards the food and when returning to the nest. The less than complete drop in arrival was likely due to the ability of the ants to recover their normal ability to sense and follow the recruitment trail in clean air. A few ants were able to follow low concentration synthetic trails despite exposure seconds earlier to a 550-fold greater concentration of pheromone, which disrupted trail following behaviour. Trail pheromone disruption of fire ants has potential for fire ant population reduction if low cost commercially available disruptants become available. Structure-activity studies and biorational design of suitable analogues / homologues that overcome trail pheromone sensitivity would be a next step.

Key words: Trail pheromone disruption, pest management, invasive species, foraging, Z,E-α-farnesene, *Solenopsis invicta*.

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Introduction

Ants comprise 5% of the world's 100 worst invasive alien species. Seventeen of the 100 invasive species are terrestrial invertebrates and 29% of these are ants, including the red imported fire ant, *Solenopsis invicta* (see LOWE & al. 2000). Currently, imported fire ants infest over 140 million hectares in the USA and cost the country an estimated 6.5 billion dollars annually for control, agriculture losses, and repair of damage to households and several other economic sectors (CORE 2003, LARD & al. 2006). In the last decade, *S. invicta* has emerged as a global pest, with new infestations established in Australia (HENSHAW & al. 2005), Taiwan (CHEN & al. 2006), mainland China (ZENG & al. 2005), Mexico (SÁNCHEZ-PEÑA & al. 2005), and many Caribbean Island countries (DAVIS & al. 2001). In addition to the above, *S. invicta* negatively affects wildlife and farm animals, interferes with biological control efforts, and about 5% of the human population in infested areas develop hypersensitivity to fire ant venom (RHOADES & al. 1989, EUBANKS 2001, ALLEN & al. 2004, PORTER & al. 2013). Although the fire ant recruitment, alarm, and queen recognition pheromone systems have been partly elucidated over the last few decades (VANDER MEER & ALONSO 1998, VANDER MEER & al. 2010), the pest control paradigm for fire ants and ants in general, remains largely confined to improving the performance of toxic baits (LOFGREN 1986, RUST & al. 2004) that use less insecticide than other methods. Ant eradication efforts also largely rely on toxic baits (HOFFMANN & al. 2011). There is a need for new socially-

acceptable fire ant eradication tactics, as well as methods to achieve long term pest management, including classical biological control (OI & al. 2015).

New methods and strategies, including technologies that deliver behavioural control through pheromones against invasive pest ants, are needed to reduce our reliance on insecticides, especially in sensitive ecosystems or where classical toxic baits are otherwise undesirable. Some progress has been made using communication disruption for the control of the invasive Argentine ant, *Linepithema humile* (see SUCKLING & al. 2008, TANAKA & al. 2009, NISHISUE & al. 2010, SUCKLING & al. 2010a, SUNAMURA & al. 2011). The advanced progress is in part due to the commercial availability of an important component of the trail pheromone for research, which happens to be also a moth pheromone (EL-SAYED 2015). Ecosystem-level interactions were recently reported, whereby ants normally dominated by the invasive Argentine ant were more competitive against it in the presence of trail pheromone disruption of the invader (WESTERMANN & al. 2014). Such ecological restoration of competitiveness could help the biotic resistance of Argentine ant-affected ant species, and we suggest that the same concept could apply to fire ants, if the novel method of trail pheromone disruption can be achieved.

Fire ant recruitment to food resources has been well defined behaviourally (WILSON 1962) and the associated pheromones have been partially identified and partitioned into three behavioural categories: A) orientation (VANDER MEER & al. 1981); B) attraction (VANDER MEER & al. 1988); and C) orientation induction (VANDER MEER & al. 1990). A scout lays a chemical trail back to the colony where it then uses relatively high concentrations of recruitment pheromone to attract and hyperactivate other workers to follow the low concentration of recruitment pheromone used to lay the trail. The movement back and forth along the trail is attributed to Z,E-α-farnesene, which is the major recruitment pheromone component. Fire ant workers recruited to follow a new trail and those already following a trail are following Z,E-α-farnesene. The use of the trail orientation pheromone to disrupt red imported fire ant resource recruitment activities is at a preliminary stage and has only been investigated in two laboratory studies using paper or aerosol delivery of trail orientation pheromone, Z,E-α-farnesene (SUCKLING & al. 2010b, 2012). A method for the disruption of fire ant communication could reduce fire ant success at foraging and provide a novel way to reduce population densities through restriction of food resources – another tactic to add to the current integrated pest management toolbox.

Here, we determine the effect of upwind oversupply of trail orientation pheromone on the time taken by foraging red imported fire ants to discover and exploit baits, in order to show the potential impact of pheromone disruption on foraging in fire ants. Z,E-α-farnesene was either applied as a point source or linear source parallel but upwind of the trail, using a similar method to that reported on Argentine ants (SUCKLING & al. 2011).

Materials and methods

Origin and maintenance of ant colonies: Fire ant colonies were reared from newly-mated queens obtained Gainesville, FL, USA, known to be monogyne (single queen) populations, based on aggression bioassays and genetic ana-

lysis (MOREL & al. 1990, VALLES & PORTER 2003). All colonies were provided with crickets, water and 10% sucrose solution, and maintained in the laboratory under ambient conditions. Ten monogyne queen-right ant colonies (fully functional with queen, workers, and brood) were used in the experiments described below. These colonies had water access, but no food was available for at least 48 h before the start of experiments to encourage foraging. For one hour before experiments, the colony to be tested was connected to a foraging arena to induce foraging behaviour. After one hour, ants were returned to their nest and a new clean arena was connected to the colony and the trial was conducted.

Chemicals and synthesis: The preparation of E,E-α-farnesene from 60 kg of cv. Granny Smith apples (*Malus domestica* × *M. sylvestris*) was accomplished following the method of MURRAY (1969). The product E,E-α-farnesene was photoisomerised to obtain Z,E-α-farnesene following RAMAIAH & al. (1995). The product obtained was a mixture of Z,E and E,E isomers 97 : 3, as determined by gas chromatography-mass spectrometry. The yield was 136 mg. The Z,E-α-farnesene was diluted in ethanol for use. Three other unknown compounds were also present in the solution at approximate concentrations of 4, 6 and 7% of the mixture (possibly including the other two isomers E,Z and Z,Z; these compounds have activity, but orders of magnitude less than that of the Z,E-isomer). For Experiment 2, remaining material after Experiment 1 was diluted from 97 to 92.5% Z,E isomer by adding E,E isomer (to increase the volume and enable an increase in replication (activity was previously recorded down to 91% purity Z,E-α-farnesene). Materials were stored at -20 °C between experiments.

Bioassays and digitisation: Z,E-α-farnesene was used to lay a synthetic 28-cm long straight trail at 10 ng / cm following the procedure in SUCKLING & al. (2012) on a glass plate (500 × 200 × 5 mm), tracing a faint pencilled line ruled on paper attached to the underside of the glass plate. Thawed cricket leg parts were used as a food stimulant at the end of synthetic trails, since these could be readily removed by individual ants. A colony of the red imported fire ant, *Solenopsis invicta*, was connected to the trail via a wire bridge to the opposite end of the trail from the cricket legs, 20 mm out of the video shot, placed on the synthetic trail leading to food. Trails were filmed diagonally across the screen from overhead using a webcam (720 by 960 pixels) and the X - Y positions of ants digitised at 15 frames per second using MaxTraq v1.92 (Innovision Systems, Lapeer, MI, USA). Movies were of 10 min duration.

Experiment 1: point source disruption: Two quarter pieces of filter paper (90° arc × 25 mm radius) were placed using a straight edge on each side of a synthetic trail at a distance of 5 mm from it, with untreated paper (control) or the treatment with 300 µg Z,E-α-farnesene on each paper point source (Fig. 1). Airflow approx (0.3 m / s) was at 45° across the synthetic trails (10 ng / cm, as above) towards a fume hood operating ~ 1 m away. The first five ants arriving from each nest into the experimental arena were digitised for five replicate nests (n = 25 ants per treatment). Two measures of behaviour were recorded: Time for bait discovery and trail integrity (R^2) of ants moving towards the food source along the straight synthetic trail (see below). Trail information from the five ants from each
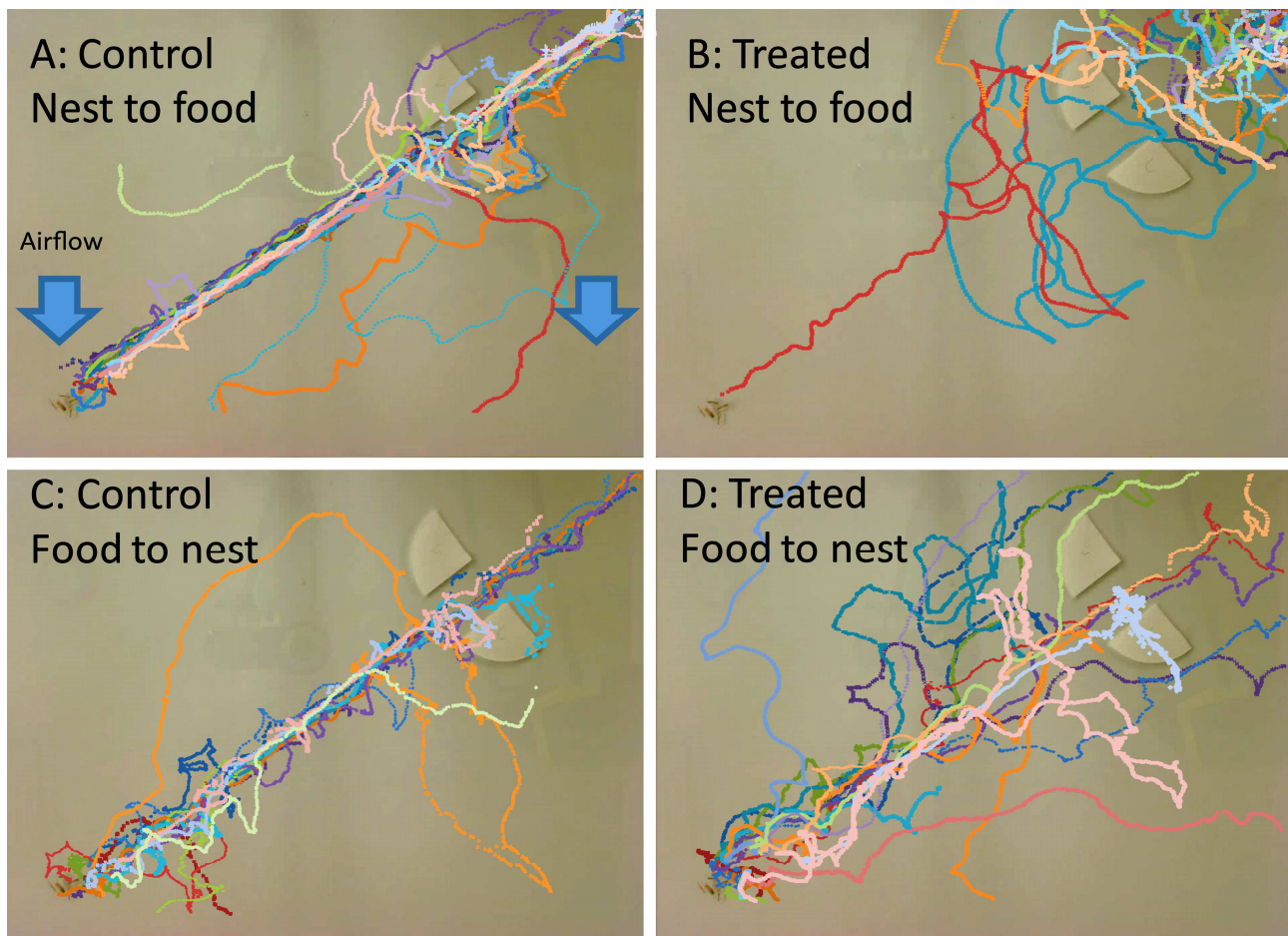
Fig. 1: Effect of point sourced trail pheromone disruption with *Z,E*-α-farnesene on foraging of *Solenopsis invicta*, for ants following a trail from the nest (top right corner of Figures A and B) to the thawed cricket bait or returning to the nest after visiting the thawed cricket bait, usually carrying it (C and D). Right side has treatment, left side has control. Airflow in all from top to bottom. Sample sizes in Table 1.

nest was pooled, since nests were the replicate unit, N = 5 per control / treatment.

Experiment 2: linear source disruption: A strip of bond paper (280 mm long × 10 mm wide) with or without *Z,E*-α-farnesene added was placed 30 mm upwind of and parallel to a synthetic trail with the same operating conditions as above. A disruptive sample of *Z,E*-α farnesene (> 92.5% purity) of 100 µl was applied to the paper strip at 10 mg / ml (5.5 µg / cm), or a 550-fold higher amount than in the synthetic trail located 30 mm downwind of the paper strip (Fig. 2). Previous results with this species (SUCK-LING & al. 2010b) (and Experiment 1) have indicated local disruption from the point sources at 300 µg loading. Four measures were recorded for ants that arrived from the wire bridge onto the trail: a) Time to arrival, b) Time to first bait removal, c) Proportion of 25 ants returning to the nest, and d) Trail integrity (R^2) of the same 25 ants (first five ants from each movie per treatment and one movie for each of five nests per treatment; therefore, n = 25). Ants randomly walking through the observation area were not analysed. The ten movies were each of 10 min duration. Since the results were consistent, a 40 s sample of trail following from each of an untreated nest (0 - 40 s of 10 min) and a treated nest (120 - 160 s of 10 min) can be viewed in Video S1 (as digital supplementary material to this arti-

cle, at the journal's web pages). The footage of the treated nest was digitised for illustration after a 120 s delay because of insufficient ants in the first 40 s.

Statistical analysis: The experimental designs used nests as units of replication, after pooling of trail information from the five ants from each nest, since trail following ants from the same nest were presumed not to be independent. The trail integrity statistic obtained by linear regression of digitised trail following ants (R^2) was calculated from the X - Y positions of each ant (SUCKLING & al. 2008) and tested for significance between treatments using Moods Median test for both experiments (Minitab 2015, v. 16.1.1), after criteria for normality in the distribution of R^2 were rejected (alpha = 0.05). The mean times for arrival at baits and bait removal were also compared between treatments and controls by *t*-test (data met assumptions of normality but were log transformed to give homogeneous variances for both experiments). For Experiment 2, the reference zone, RZ, a statistically and spatially-defined location of where the ants entered by trail into the movie frame, was calculated by taking the mean X - Y ± 1 SD pixel position in the x and y plane of the control ants when they first entered the frame on their outward journey from the nest. The mean ± 1 SD represents 68% of the position of ants that would be expected to cross this
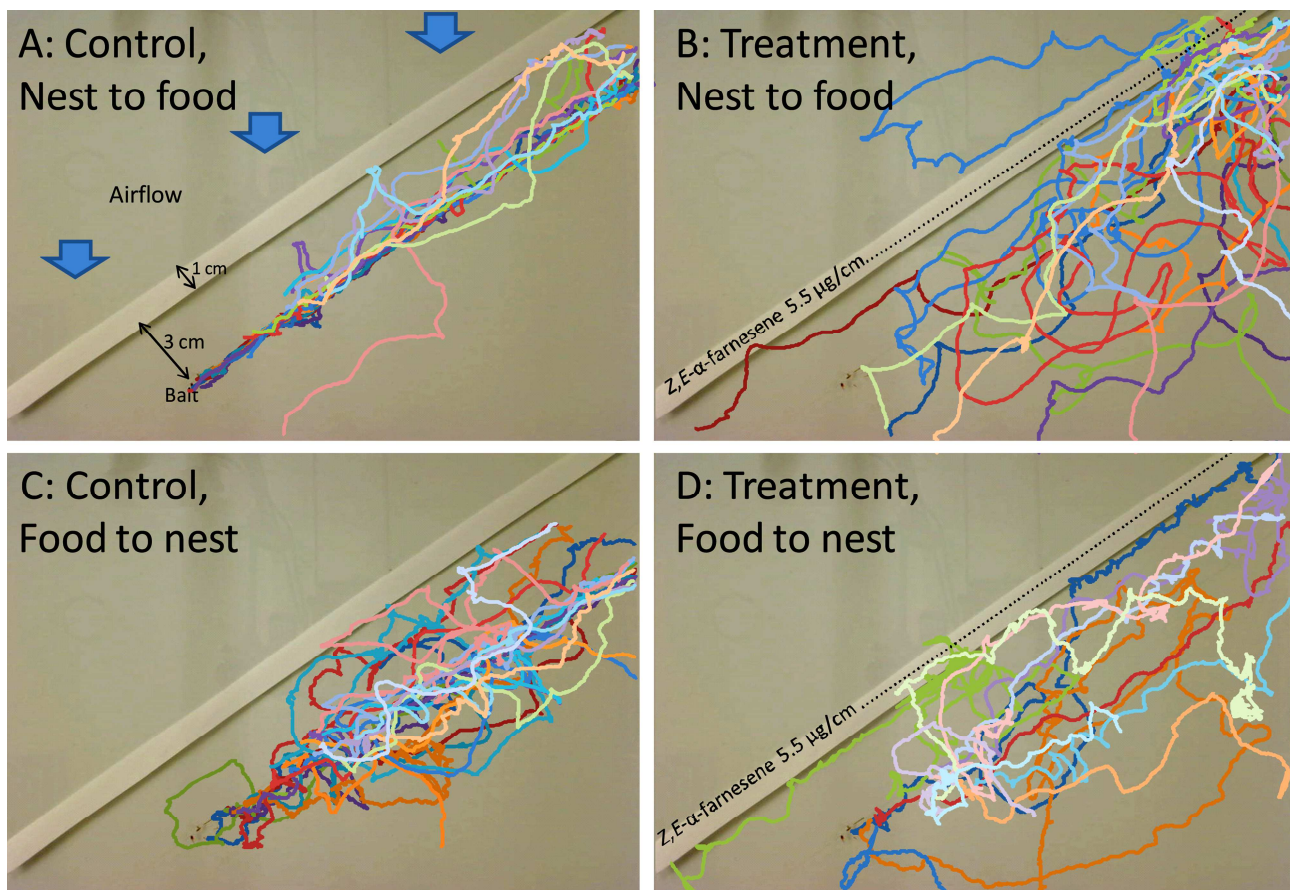
Fig. 2: Visualisation of trail pheromone disruption with Z,E-α-farnesene on foraging of *Solenopsis invicta*, for ants following a trail from the nest (top right) to the thawed cricket bait (A and B) and returning to the nest after visiting the thawed cricket bait (C and D). Airflow in all from top to bottom. Sample sizes in Table 2.

position enroute to the nest, determined from the earlier ant-defined areas in the controls. For the ants returning to the nest with food, the number of individuals that left the movie frame within the RZ of the control was counted in both the controls and treatments, since it was hypothesised that in the absence of any effect the normal trail following ants should cross this area. This was changed into a proportion (N leaving within area / total N) and this proportion was multiplied by 68% (to give the expected number crossing RZ) and the resulting proportional success rate was compared with a X^2 test, in order to test this hypothesis.

Results

Experiment 1: point source disruption: Without communication disruption, ants formed distinctive trails along the synthetic trail drawn between the nest and food (Fig. 1A), although some ants were apparently distracted by the nearby upwind and downwind paper quarter circles, leaving the trail and investigating them. Trail following ants returning with cricket parts (Fig. 1C) also trailed to either side of the synthetic trail, with more looping back to it than was evident on the way from nest to food (Fig. 1A).

The ability of the ants to form trails and specifically the trail integrity statistic was significantly reduced in the presence of the point source disruption treatment (Tab. 1), according to non-parametric tests. One ant in particular (Fig. 1B) clearly regained the synthetic trail to the food after passing of the downwind of the treated paper quarter

Tab. 1: Moods Median Test of trail integrity measured as r^2 for red imported fire ant (*Solenopsis invicta*) tracks when walking in the presence or absence of excess Z,E-α-farnesene from a point source (first five ants from each of five nests departing the nest and going to the food source pooled, so that nests were taken as replicates, n = 5).

Parameter	Control	Pheromone-treated	X^2	P
Median r^2	0.920	0.263	10.0	0.002
Q3-Q1	0.205	0.128		
n	5	5		

Tab. 2: Moods Median Test of trail integrity measured as r^2 for red imported fire ant (*Solenopsis invicta*) walking tracks with or without excess Z,E-α-farnesene from a linear source upwind (first five ants from each of five nests averaged and taken as replicates, n = 5 nests), data reduced from 25 to 13 for pooling where ants were completely disrupted.

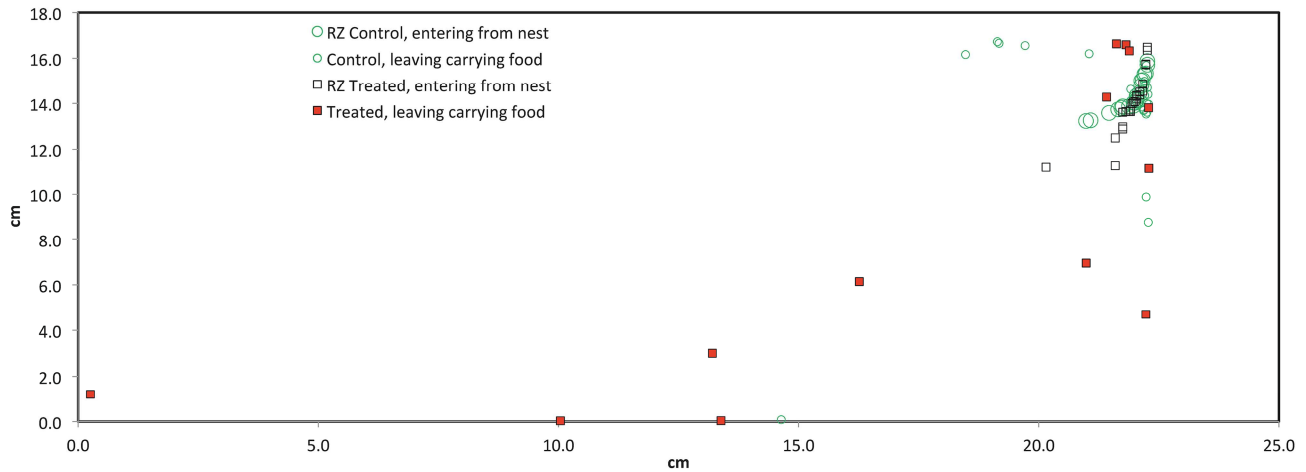| Direction	Parameter	Control	Pheromone-treated	X^2	P
Nest to food	Median r^2	0.823	0.630	1.10	0.294
	Q3-Q1	0.324	0.477		
	n	5	5		
Food to nest	Median r^2	0.937	0.381	10.0	0.002
	Q3-Q1	0.091	0.165		
	n	5	5		

Fig. 3: The reference zone (RZ) generated by location of incoming control *Solenopsis invicta* workers from a nest into the video frame (n = 5 nests) arriving into the bioassay along a synthetic trail towards a thawed cricket bait; this is the basis for comparison with ants departing the frame with cricket parts, in the control and treated areas.

circles. Similarly, three ants were successful at trail following on the return from the food, including near the treated paper upwind (Fig. 1D), although most of the ants were disrupted throughout the observation period. A 2.4-fold increase in the mean time for ants to locate the thawed cricket legs was observed in the presence of the trail pheromone treatment (Control: 36.6 ± 6.2 s, Treatment 121 ± 18.7 s; $t_7 = 4.95$, $P = 0.002$).

The videos revealed evidence of a response of some ants to the papers in the controls (deviation to the nearby control point source papers on both upwind and downwind sides of the trail, followed by edge following along the paper shape Fig. 1), which was undesirable since it affected the baseline of trail following to the odour and the trail integrity statistic. This led to a modified setup in Experiment 2, with a linear source of disruptant upwind of the synthetic trail.

Experiment 2: linear source disruption: Ants that returned to the colony after visiting food in the absence of treatment had lower trail integrity than usually observed on the way out (Tab. 2), and the treatment values for r^2, although lower again, showed no significant difference from the control as a result (Time to bait discovery, Control: 53.0 ± 13.1 s, Treatment 95.2 ± 20.2 s; $t_7 = 1.69$, $P = 0.135$); Time to bait removal, Control: 96.2 ± 16 s, Treatment 264.4 ± 62.0 s; $t_7 = 3.26$, $P = 0.01$). This represents a 1.7-fold increase in the time to bait discovery and a 2.75-fold increase in the time to bait removal.

A behavioural difference is evident, when the individual tracks are observed directly (Fig. 2).

The proportion of the ants returning to the nest was also affected by the trail disruption (24 / 24 returned in the controls and only 13 / 25 (52%) returned in the treatment). However, the treatment included a very few ants which were successful at discovery of the trail after entering clean air from the area with the upwind disruption treatment.

The ability of ants to locate the trail again and follow it, after experiencing the trail pheromone disruption treatment was investigated with another method. The analysis of ants crossing the Reference Zone (RZ) on their way back to the nest, which was determined from trail following ants departing the nest in the control, showed that the

probability of the 24 returning ants crossing this area was 68% for the control, the expected value, while for the treatment this probability (n = 13) dropped to 22.7% (Fig. 3). This difference was statistically significant using a Yates correction (z = 2.218, $P = 0.027$). The spread of the untreated insects (green) on returning to the nest via the wire to the right of the frame was greater on their return trip due to transport of prey items. The walking tracks of returning ants using the trail showed scalloping patterns as they dragged the cricket pieces but responded to the trail (Fig. 3). The disrupted ants showed a wider spread of departure points and end points. Some ants returned to the trail upwind of the disrupted area suggesting that they had recovered from the effect of the pheromone treatment.

Discussion

The *Z,E-α*-farnesene trail pheromone component of the red imported fire ant (which so far has only been deployed in laboratory experiments) clearly has the ability to reduce foraging by worker ants, at least at close range, and the concept represents a new prospect for behavioural control of ants which will require a directed and targeted effort to find more active materials as well as more practical ways of achieving behavioural disruption in ants.

Once the fire ants have located the food, their ability to make it back to the colony was reduced, but was not completely eliminated in these studies. This could be related to the high density of ants resulting from proximity to the highly populated laboratory colonies and / or the small arena size. The limited size of the arenas undoubtedly raised the probability of chance encounters with the trail in clean air at the RZ. The situation could be different at the natural landscape scale. Visual cues may have been used by ants visiting both upwind and downwind paper quarters. Airflow disturbances detectable by the ants could explain upwind foraging with thigmotaxis to the paper edge on arrival, but downwind foraging from the trail is harder to explain.

Our previous examination of the change in turning angles after exposure (SUCKLING & al. 2012) illustrates the mechanism by which trail pheromone disruption is probably operating. Ants normally criss-cross the trail (HANGART-NER 1967) with a narrow range of track angles (see Video

S1), but the sudden shift to a much flatter track angle induced by the treatment in walking ants (SUCKLING & al. 2012) is probably caused by the lack of a clean air signal to turn at the right time. Affected ants consequently show meandering or sometimes circular paths in the treated area (Figs. 1, 2, and Video S1). This rarely happens in the untreated areas, although on the way back to the nest some ants occasionally struggle to follow the trail while carrying food.

In the controls for Experiment 1, some ants deviated from the trail to investigate nearby (5 mm) paper quarter circles which had been only solvent treated, but most rejoined the synthetic pheromone trail. Because this effect occurred both upwind and downwind of the synthetic chemical trail, this led us to consider that visual clues from the paper were probably responsible for the deviation (as opposed to mechano-reception of local airflow variations on the downwind side which would be unlikely to be detected). The second experiment was designed to reduce this effect with a linear rather than a point source dispenser upwind. The RZ, or area described the point of entry of the individual ants into the frame provided a valuable basis for comparison with the point of departure, which was distributed nearby the entry RZ for the controls, but not for the treated ants where the values were highly variable.

The ants showing recovery of the trail, away from the influence of point sources corroborate observations in *Linepithema humile* of the lack of an effect of habituation, despite the high trail pheromone concentrations experienced by the disrupted ants. The fire ants do not appear to show any long lasting effects of 550-fold higher pheromone concentrations than we used in the synthetic walking trails, which is in contrast to pre-exposure of moths to sex pheromones, where a lack of mating behaviour can result for many hours following exposure of single female equivalents (BARTELL & LAWRENCE 1973). The trail pheromone for the Argentine ant, *Linepithema humile*, the only commercially available pest ant trail pheromone, has recently been investigated in field trails in vineyards where the economic problem is Grapevine Leafroll Virus A vectored by a mealybug tended by the ants (WESTERMANN & al. 2014). This work is proving effective but not cost effective so far because of the similar need for continuous treatment. Currently, the lack of a commercial supply of Z,E-α-farnesene hinders its further development as a novel fire ant population suppression method (SUCKLING & al. 2012), but the amount of material required and the immediate recovery outside the treated area also likely present major barriers, because of sensitivity and reversibility of the olfactory receptor neuron responses.

However, the fire ant exerts large economic effects on the countries it currently infests and global spread with trade into new locations will increase pressure to develop novel, environmentally friendly methods for control. Our preliminary studies on trail pheromone disruption of fire ants demonstrate potential as a method to suppress foraging and thereby populations, for a second species of ants. Better new synthetic methods of communication disruption are needed to progress the concept practically against fire ants. Perhaps this could involve analogues with longer lasting and more sensitive effects. Ideally, these types of solutions could be developed, to overcome the current practical limitations of the natural product. So far, the mechanism of change in individual ant trail following behaviour appears to come from the removal of the ability of the ant to discriminate information about the boundary of the trail and clean air and turn appropriately to ensure trail following. Breaking this code actually represents a step towards enabling more practical measures, since it apparently amounts to a chink in the armour of the fire ants. It is already clear that control using this type of technology for disrupting any ant pest will be very challenging without a low cost commercial supply of active pheromone compounds or a good way of delivering their disruption on the landscape. Structure-activity studies and biorational design of suitable molecules to overcome recovery of trail pheromone sensitivity at the olfactory receptor neuron level would appear warranted as a next step.

Acknowledgements

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