Trail Pheromone Disruption of Red Imported Fire Ant

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Abstract The fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), is considered one of the most aggressive and invasive species in the world. Toxic bait systems are used widely for control, but they also affect non-target ant species and cannot be used in sensitive ecosystems such as organic farms and national parks. The fire ant uses recruitment pheromones to organize the retrieval of large food resources back to the colony, with Z,E-α-farnesene responsible for the orientation of workers along trails. We prepared Z,E-α-farnesene, (91% purity) from extracted E,E-α-farnesene and demonstrated disruption of worker trail orientation after presentation of an oversupply of this compound from filter paper point sources (30 μg). Trails were established between queen-right colony cells and food sources in plastic tubs. Trail-following behavior was recorded by overhead webcam, and ants were digitized before and after presentation of the treatment, using two software approaches. The linear regression statistic, r^2 was calculated. Ants initially showed high linear trail integrity ($r^2=0.75$). Within seconds of presentation of the Z,E-α-farnesene treatment, the trailing ants showed little or no further evidence of trail following behavior in the vicinity of the pheromone source. These results show that trailing fire ants become disorientated in the presence of large amounts of Z,E-α-farnesene. Disrupting fire ant recruitment to resources may have a negative effect on colony size or other effects yet to be determined. This phenomenon was demonstrated recently for the Argentine ant, where trails were disrupted for two weeks by using their formulated trail pheromone, Z-9-hexadecenal. Further research is needed to establish the long term effects and control potential for trail disruption in *S. invicta*.

Key Words Red imported fire ant · Ant · Trail pheromone · Trail disruption · Invasive species · Z,E-α-farnesene · *Solenopsis invicta*

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Introduction

In the early 1960s, Wright proposed that synthetic sex pheromone could disrupt mating of moths (Wright 1964). The feasibility of this was soon demonstrated (Gaston et al. 1967). Since then, the identification of sex pheromones of many moth species has been achieved along with the development of successful direct pest control options based on pheromones and other attractants (El-Sayed 2010; Witzgall et al. 2010). Despite the discovery and characterization of ant trail pheromones over the past several decades (Tumlinson et al. 1971; El-Sayed 2010), surprisingly little examination of these compounds has been undertaken for pest management. Research on the potential for using odorants in this way has targeted the control of leaf cutting ants (Robinson and Cherrett 1978) and the red imported fire ant (Vander Meer 1983, 1996), but the current paradigm remains largely confined to improving the performance of toxic baits (e.g., Rust et al. 2004).

New application technologies that deliver pheromones against invasive pest ants could help reduce our reliance on

the use of insecticides for ant pest control in sensitive ecosystems or where insecticides are undesirable. Trail pheromone disruption that affects recruitment is an example of a novel tactic for ant pest management. The Argentine ant (*Linepithema humile*) trail pheromone, Z-9-hexadecenal (Cavill et al. 1979), is being examined for disruption of trails by using either polyethylene tubing dispensers (Tatsuki et al. 2005; Tanaka et al. 2009) or widely dispersed point sources of pheromone (Suckling et al. 2008, 2010). This compound also is a moth pheromone (El-Sayed 2010). Tubing dispensers showed some disruption of traffic rates on the ground or tree trunks, when placed on bamboo at a 50 cm height above the ground (Tatsuki et al. 2005). The mechanism of disruption of trailing behavior observed for individuals at close range before and after exposure involved changes in walking track angles (Suckling et al. 2008). This single component was shown at a laboratory scale to be capable of disorienting trail following workers when presented in greater than physiologically normal quantities (Suckling et al. 2008). Measurement of trail disruption was achieved by assessing trail linearity as measured by the square of the linear correlation coefficient (r^2) (Suckling et al. 2008). The disruption concept was extended successfully to the field by using widely-dispersed wax-covered sand laden with the pheromone (Suckling et al. 2008) and formulated as sprayable microencapsulated particles (Suckling et al. 2010). Treatment achieved up to 90% disruption of visible Argentine ant trails for two weeks in 400 m^2 plots under hot and windy conditions in Hawaii's Volcanoes National Park (Suckling et al. 2010). Foraging to baits also was significantly reduced. These interesting developments suggest that this approach could have applicability to other invasive pest ant species, if impacts on nests can be demonstrated.

The Red Imported Fire Ant, *Solenopsis invicta*, is one of the 100 worst invasive alien species in the world (Lowe et al. 2000). Imported fire ants infest more than 330 million acres in 13 southern tier US states and Puerto Rico and are spreading northward (USDA APHIS 2009). This invasive ant is estimated to be responsible for almost $7 billion annually in damage repair, medical care, and control costs. The affected economic sectors are broad ranging and include households, electric service and communications, agriculture, schools, and recreation areas (Lard et al. 2006). In the last decade, *S. invicta* has changed from an invasive pest ant in the United States to a global problem, with infestations occurring in New Zealand (now eradicated, Christian 2009), Australia (Henshaw et al. 2005), Mexico (Sánchez-Peña et al. 2005), Taiwan (Chen et al. 2006), China (Zhang et al. 2007), and many Caribbean Island countries (Davis et al. 2001). The monogyne social form of *S. invicta* in the USA has population densities up to 100 colonies/ha and, at 250 thousand workers per colony, the resource demands are great.

Toxic ant baits have been developed and refined over the past decades in response to a need for fire ant control over large areas with minimal amounts of toxicant (Williams et al. 2001). Toxic baits are more environmentally friendly in that they introduce significantly less insecticide into the environment than mound drenches. However, fire ant baits also have a negative effect on non-target ant species that could otherwise slow fire ant reinfestation rate by feeding upon newly mated fire ant queens (Nichols and Sites 1991). More environmentally-friendly control methods are needed for management and, under certain circumstances, eradication of this invasive ant. Disruption of the critical recruitment process potentially could decrease colony resources and concomitantly population densities, and/or induce colonies to move.

Ants exhibit a wide variety of recruitment and orientation methods by using pheromones that direct worker movement between the colony and food sources or during colony migration. Similarly, the glandular sources and the chemistry of recruitment pheromones is diverse (Vander Meer and Alonso 1998). The Dufour's gland of fire ant workers is the source of its recruitment pheromone, which has been divided into three behavioral categories: orientation induction (Vander Meer et al. 1990), attraction (Vander Meer et al. 1988), and orientation (Vander Meer et al. 1981). Orientation induction requires essentially all Dufour's gland components, and acts to increase the sensitivity of workers to the orientation part of the pheromone. Two components, Z,E-α-farnesene and a bicyclic homosesquiterpene-diene, homoeudesmandiene, but not a tricyclic homosesquiterpene monoene as previously suggested (Vander Meer et al. 1988), account for 100% of the attractive element of the recruitment pheromone. Z,E-α-Farnesene accounts for 100% of the orientation (movement of worker ants back and forth along a trail) part of the recruitment process. The ants are sensitive to Z, E-α-farnesene trails as low as fg/cm levels. Laboratory trials were the first step in assessment of the potential for developing trail pheromone disruption against the Argentine ant (Suckling et al. 2008), before more expensive field trials (Suckling et al. 2010). Since behavioral responses to synthetic chemically-based disruption are concentration-dependent, we considered that it was sufficient to record statistical significance with the phenomenon in the laboratory as a first step before formulation development. This paper focuses on demonstration of the potential for trail pheromone disruption of red imported fire ants by using Z,E-α-farnesene, the recruitment orientation pheromone.

Methods and Materials

Chemicals and Synthesis A mixture of Z,E-α-farnesene (91% purity) and E,E-α-farnesene (9%) was synthesized

and maintained in a petroleum ether (115 mg/ml) stock solution until use. The preparation of E,E-α-farnesene from 60 kg of cv. Granny Smith apples (*Malus domestica × M. sylvestris*) was done following Murray (1969). The 60 kg of apples were divided into 5×12 kg batches that were washed, adding the apples in portions in 2 L of petroleum ether (boiling range 35–60°C). The apples were stirred for approximately 1 min then transferred into another 1.5 L of solvent where the stirring process was repeated. The solvent from the combined extracts of all the apples was removed *in vacuo* to give 36.2 g of a waxy solid. The solid was analyzed by TLC (10% EtOAC, 90% petroleum ether), which indicated the presence of E,E-α-farnesene by comparison with an authentic standard. The waxy solid was then dissolved in 400 ml petroleum ether (boiling range 35-60°C) and refrigerated overnight at 0°C. Solids were removed by filtration and discarded. The solvent extracts then were divided into two equal portions, and to each portion urea (130 g) was added. The material was refrigerated again overnight at 0°C. Solids were removed by filtration, and the solvent removed *in vacuo*. The crude product thus formed was purified by column chromatography using 5% EtOAc / 95% petroleum ether as eluant. The appropriate fractions were collected and repurified under the same conditions. The product (1.05 g), verified by ^{1}H-NMR (Spicer 1994), was collected as a colorless oil.

Following the procedure of Ramaiah et al. (1995), photo-isomerization of E,E-α-farnesene to Z,E-α-farnesene entailed dissolving E,E -α-farnesene (385 mg, 1.88 mmol) in benzene (12 ml), and dividing the solution into 4×3 ml portions that were put into 4 quartz spectrophotomer cells $(1 \times 1 \times 4$ cm). A drop of acetophenone was added to each of the samples. The solutions were sealed with a plastic lid and then photo-irradiated at 254 nm using a Hoefer UVC 500 UV Crosslinker. After 4 h, the photo-isomerization had reached approximately 40% conversion, and further irradiation had no effect. The solvent then was removed *in vacuo*, the crude material was redissolved in diethyl ether (10 ml), and tetracyanoethylene was added in small portions (50 mg at a time) until monitoring by GC-MS indicated that most of the unwanted E,E isomer had been consumed. It was not possible to remove all of the E,E isomer by addition of further tetracyanoethylene. The solvent was removed *in vacuo*, and the product was purified by column chromatography using petroleum ether as eluant. The product was obtained as a colorless solution, which was a mixture of Z,E:E,E isomers in the proportion 91:9 as determined by GC-MS, with a final yield of 115 mg.

Insects Queen-right colonies of *S. invicta* were collected in the field or started from field-collected newly-mated queens and maintained by standard methods (Banks et al. 1981).

Prior to experiments, a colony cell (15 cm Petri dishes with a central hole in the lid and a layer of moistened Castone® in the bottom) was placed at one end of a white plastic tub $(50 \times 37$ cm and 12 cm deep) that had the inner sides coated with Fluon® (Asahi Glass Co. Tokyo) to prevent escapes. Each colony cell was allowed to rest for at least 16 h before experiments. The ants were starved, but had continual access to water. Trail formation was achieved by placing one thawed house cricket (*Acheta domesticus*) and an absorbent cotton ball (Johnson and Johnson) dampened with 10% sugar water at the opposite end of the tub from the colony cell. Trials began once the trail had formed, with workers visibly trailing back and forth.

Video Recording A Logitech webcam $(640 \times 480$ pixels, Notebook Pro, Logitech, Freemont, CA, USA) supported by retort stand was used to record a vertical view of fire ant workers trailing to and from a food source. Filming generated Audio Video Interleave (avi) files at a rate of 15 frames/s onto a laptop computer (actual screen size 10×12 cm at 480×640 pixels) to record fire ant walking tracks, with the method developed on Argentine ants (Suckling et al. 2008).

Experiment 1 Point source trail disruption was tested by placing treated (30 μg Z,E-α farnesene in 10 μl petroleum ether) or control filter paper (10 μl petroleum ether) within ca. 1 cm of an established trail. The treatment and control solutions were applied to filter paper squares (Whatman No. 1, 1 cm²) by gas tight syringe and allowed to evaporate for ca. 1 min in a fume hood before use. The fume hood was left switched on for the entire experiment to remove any excess pheromone from the laboratory. Furthermore, control papers were tested before treated papers to reduce the likelihood of pheromone contamination. The experiment was repeated four times ($N=4$ colonies). Movies were recorded for 1 min before, and 1 min after, paper squares were placed within 1 cm of an existing trail.

Experiment 2 This experiment aimed to test the effect of trail disruption at two concentrations (solvent control plus 30 or 300 μg Z,E-α farnesene), and was repeated four times ($N=4$ colonies), using the same methods as above. Movies were recorded for 1 min before, and up to 10 min after paper squares were placed within 1 cm of an existing trail.

Statistical Analysis Two software approaches were used for track analysis, by instantaneous and continuous tracking. The instantaneous position of individual ants was recorded using MaxTraq v1.92 trial edition (Innovision Systems, Lapeer, MI, USA) (Suckling et al. 2008). The x-y positions of all the ants present were recorded from individual frames at 5 s intervals, as this was sufficient time for the ants to leave the video frame under control conditions, rendering
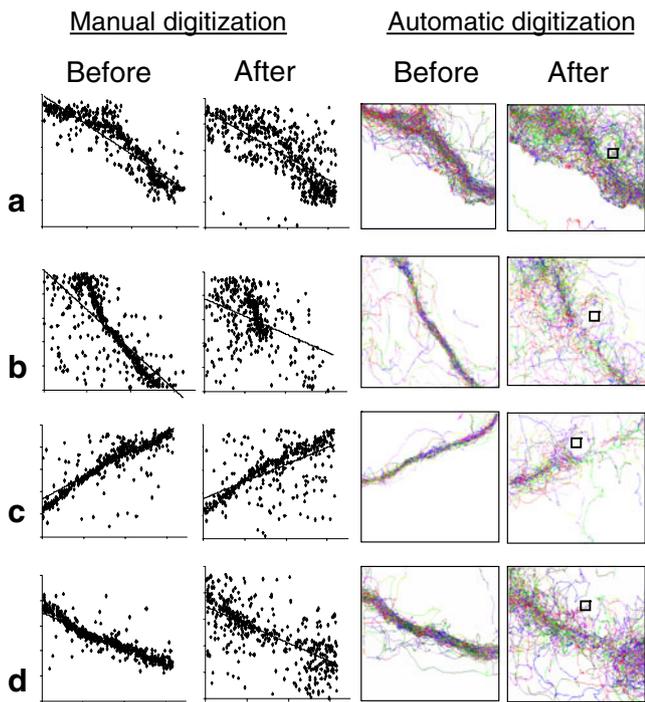
Manual digitization

Before   After

Automatic digitization

Before   After

a

b

c

d

Fig. 1 Superimposed positions of red imported fire ants, *Solenopsis invicta*, in four replicate trails (**a–d**), with either manual digitization (left two columns) sampled instantaneously at 5 s intervals from 1 min before and 1 min after treatment, or continuously using automatic digitization (right two columns). The small square shows approximate placing near the ant trail of 1 cm filter paper loaded with 30 µg Z,E-α-farnesene. Trail integrity (r^2, calculated from manual digitizations) dropped from the minute before to the minute after in each replicate (**a**: $r^2=0.77–0.49$, **b**: $r^2=0.58–0.16$; **c**: $r^2=0.72–0.40$; **d**: $r^2=0.81–0.45$)

the individual samples independent. Digitizing required one mouse click per ant, but it was possible to capture the position of every ant in the frame (a labor intensive process), which enabled the determination of the linearity of trails in each frame, by calculation of r^2.

However, it was realized that this could be replaced with an approach that sub-sampled the movements of multiple individual ants, frame by frame, for differences with the preceding reference frame.
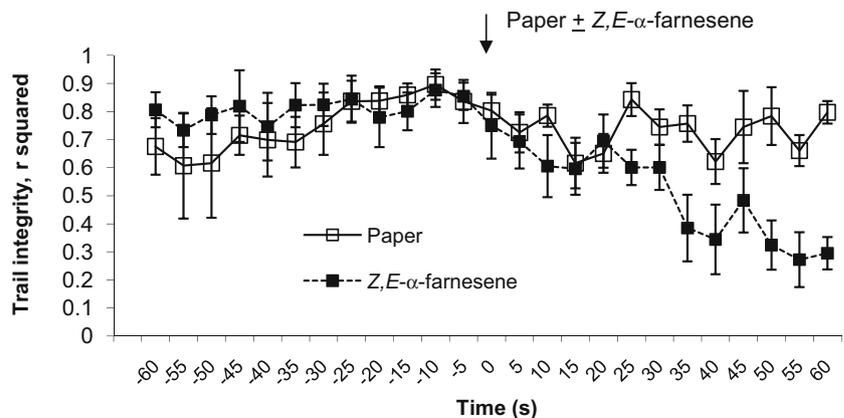
Thus, continuous analyses of the majority of ants walking (up to 25 individuals per frame) were conducted after processing, by using novel software that was developed in house with HALCON/C languages interface (MvTech Software GmbH, Munich, Germany, 2009) for the Microsoft Windows operating system. The Multiple Individual Tracking Software enables the implementation of high-speed video tracking systems capable of accurately tracking multiple objects (2–25 insects), at high speeds (>60 frames/sec). It allows for offline processing of digitally recorded videos as well as real-time processing of images from fast video streams (where the video stream does not require storage except as a text file). Identification of the target is based on both the morphological features, and its temporal speed in successive images. It utilizes a subtraction algorithm to construct tracks for each target insect. The target of the previous image is matched with the target of the current image, creating unique motion estimation for each moving object. During this process, any target estimation errors, such as when insects occlude and split, are detected and corrected. Tracking is activated once an insect enters the camera field of view. The insect is detected immediately by the change in the detection modules. While tracking insects, the tracking module keeps looking for new insects entering the field of view. The digitized images recorded per frame as X,Y coordinates for each insect were stored as a text file on the hard disk. Another program, written in Borland Delphi (version 7; Borland Software Corporation, Austin, TX, USA), was used to visualize the tracks and to export the images of resulting tracks as bitmap files. In addition, this program was used to analyze and evaluate the tracks and to calculate movement parameters.

Results

Ant trails showed clear visual evidence of a trail disruption effect well within a minute of exposure to 30 µg Z,E-α-

Fig. 2 Red imported fire ant, *Solenopsis invicta,* trail integrity (mean±SEM), r^2, sampled at 5 sec intervals for 1 min before and after introduction of 30 µg Z,E-α-farnesene (statistical significance is presented in Table 1)

Table 1 Two way analysis of variance of red imported fire ant (*Solenopsis invicta*) trail integrity (measured as r^2), 1 min before and 1 min after treatment with filter paper loaded with 30 µg Z,E-α-farnesene
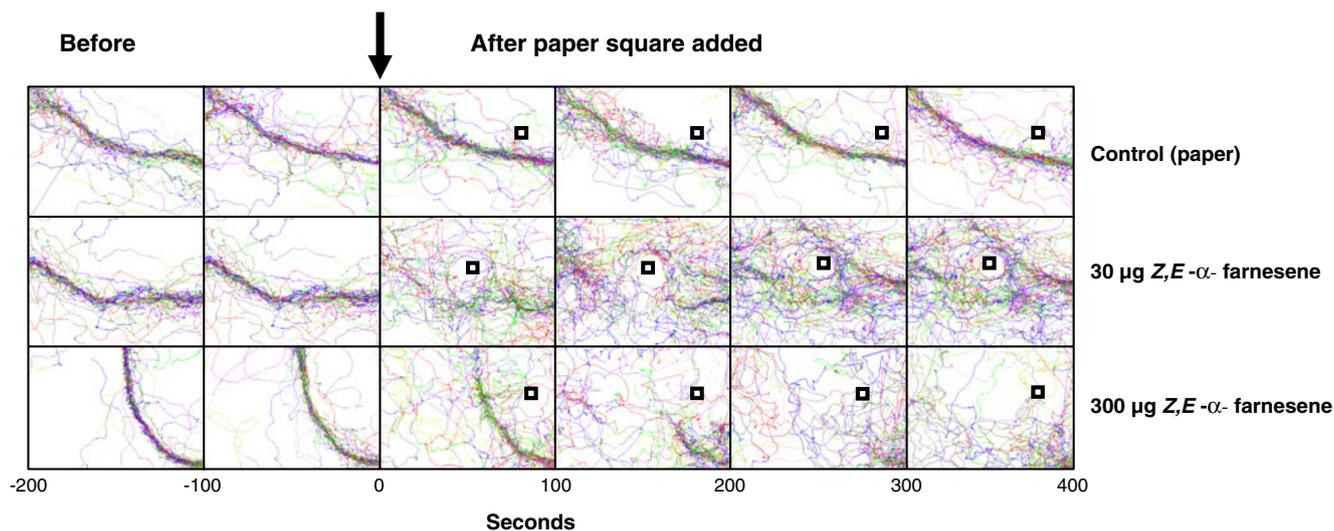
Source	DF	Sequential Sums of Squares	Adjusted Sums of Squares	Adjusted mean square	F	P
Treatment	1	0.5440	0.5951	0.5951	15.48	<0.001
Time	1	1.7389	1.4446	1.4446	37.59	<0.001
Treatment * Time	1	1.0880	1.0880	1.0880	28.31	<0.001
Error	224	8.6089	8.6089	0.0384		
Total	227					

farnesene presented as a point source on filter paper (supplementary material, downloadable movie of trail disruption and software). Two methods extracted similar behavioral effects. The width of the trail was broadened visibly in the minute after treatment, and the effect was quantified using the trail integrity statistic r^2 (Suckling et al. 2008) derived from locations of ants sampled every 5 sec for a minute before or after presentation of treated paper (Fig. 1). The software using continuous recording of the actual trails of ants showed a similar effect, but in more detail. Random movement away from the previous trail was more evident.

Obvious trails, with significant r^2 values, were evident only before treatment, and after treatment only with solvent controls (Fig. 2). With the trail pheromone treatment, the ants were less able to follow the trail they had sensed moments before, and this was demonstrated by the reduction of the r^2 value after treatment (Fig. 2). The mean (±SEM) values before treatment (r^2=0.75±0.03) were

reduced after treatment (r^2=0.48±0.03), compared to the controls that did not change (before r^2=0.75±0.03; after r^2=0.73±0.02). The two-way analysis of variance showed a significant effect from pheromone treatment and time (before and after treatment), as well as an interaction between these variables (Table 1) because of declining trail integrity over time (Fig. 2). A short delay occurred after treatment before the trail showed evidence of disruption as diffusion occurred, so assessment in the first minute is a conservative measure of an effect.

The effect of increasing disruption over time and with an increased concentration of the pheromone is evident in the next experiment (Fig. 3), displayed using the Multiple Individual Track System. When post-treatment recordings of longer duration are examined, the trails show increasing evidence of disruption over time. In the case of experiments with 300 µg loadings of Z,E-α-farnesene on filter paper (Fig. 3), the trails appear to literally melt away over time as diffusion spreads the oversupply of trail pheromone over existing trails.

Before　　　　　　　　　　**After paper square added**

Control (paper)

30 µg Z,E -α- farnesene

300 µg Z,E -α- farnesene

-200　　　　-100　　　　0　　　　100　　　　200　　　　300　　　　400

Seconds

Fig. 3 Trail formation in red imported fire ant, *Solenopsis invicta*, before treatment (-200-0 s) and at 0–400 s after trail disruption, from the addition of an oversupply of trail pheromone Z,E-α-farnesene (solvent control, 30 or 300 µg on 1 cm² of filter paper). Walking tracks of ants were recorded by webcam and analyzed by Multiple Individual Tracking Software, written in-house

Discussion

We have demonstrated for the first time that the trail-following behavior of *S. invicta* can be disrupted by an oversupply of the trail pheromone, *Z,E-α*-farnesene. This is the first step in a new line of enquiry against this pest species. The precedent case of trail pheromone disruption in one other species (Argentine ant) has advanced to a formulation lasting up to two weeks in the field under windy conditions, and field tests over several months in a combination treatment with toxic baits as part of the Japanese eradication program at the port of Yokohama (S. Tatsuki pers. comm.). A single point source can be used for demonstration of disruption of the nearest trails (Tatsuki et al. 2005; Suckling et al. 2008), although demonstration of sustained nest level effects is needed (Suckling et al. 2010). The potential to use trail disruption as a management tool or in some circumstances, eradication, against fire ants is a worthy goal, but will require suitable technology to release *Z,E-α*-farnesene or other compounds at an effective rate and cost.

The analysis of ant trail movies into statistical parameters such as r^2 for trail integrity sampled at regular intervals (Suckling et al. 2008) enabled a new approach to measuring the impact of disrupting trailing behavior. The bioassay uses a statistical definition of trail following (r^2), and trail disruption is defined when this statistic becomes insignificant. This offers a number of advantages, including an objective statistical basis and lack of bias as to what constitutes a trail, disrupted or otherwise. However, it is labor intensive. In contrast, the development of software that automatically performs the digitization of multiple individuals and tracks their movements without requiring mouse clicks offers greater digitization speed. These advanced functions expedite recording and analysis of insect behavior, and have enabled our experiments to investigate trail behavior from many individuals quickly.

Trail pheromone disruption of *Solenopsis invicta* warrants further examination, but any eventual use would rely on appropriate technology for delivery of the necessary concentrations, as well as an understanding of the biological implications, if any, of reducing the efficiency and potentially information and energy (food) content of workers returning to the nest. The chemical instability and likely cost of the trail pheromone compounds could prove to be an insurmountable problem to achieving this, although further research may overcome these barriers, particularly since plant-based production via synthase has been established (Green et al. 2007). If this is the case, there could be various ways of using the phenomenon of trail disruption, depending on how well it works and what effect it has on the insects over time. There are also other related ant species which use this compound (Barlin et al. 1976; El-Sayed 2010). It is potentially useful in integrated pest management, for example in combination with poison baits, possibly before baiting treatment to improve bait removal to the nest after starvation, or after insect densities have been lowered by baiting. Trail disruption could prove to be useful in the event of detection of new incursions of this species, for example to island nations (Davis et al. 2001; Henshaw et al. 2005; Chen et al. 2006; Christian 2009), where the prospects of eradication may be greater.

Certainly, there is a high risk of failure in establishing an entirely new pest management tactic against ants based on trail pheromones, but there is also a great reward for success if they can be successfully developed and deployed for these pests. The very low eco-toxicological hazard of these compounds presented in such a low quantity means that they could become acceptable tools for use in sensitive environments, and wherever conventional pesticides are undesirable. We have shown that a trail pheromone compound can locally disrupt *S. invicta* trails, and other active compounds should also be investigated for their potential effects. Trail pheromone disruption against this species will require the development of sustained release formulations to determine if the approach can be made practical in the field.

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