Hawaii Invasive Species Council (HISC) Research and Technology Grant Program, Final Report

Project Title: Chemical ecology of the Little Fire Ant, *Wasmannia auropunctata* (Roger), for detection, delimitation and control in Hawaii

Project Number: 58148

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Executive Summary: The central objective of the research described here was to develop tools to detect, delimit and control the little fire ant (LFA), *Wasmannia auropunctata* (Roger), in Hawaii. Current detection methods rely mainly on peanut butter and other general food baits which are not specific for LFA. Alkylpyrazines were found to constitute the major volatiles released from peanut butter when assayed by head-space using solid phase microextraction (SPME) and analysis by gas-chromatography/mass-spectrometry (GC/MS). Field testing of these compounds showed little or no attraction. Likewise, laboratory bioassays did not show strong attraction to peanut butter over Spam baits and suggest that ant foraging behavior, not volatile attraction, are responsible for bait discovery.

Given these disappointing results, we turned our attention to ant-produced pheromones, which showed much higher levels of attraction. GC/MS analysis of ant extracts was used to identify two compounds, 2,5-dimethyl-3-(2-methylbutyl)pyrazine and 3-methyl-2-(2-methylbutyl)pyrazine. Headspace sampling of confined ants with SPME and Porapak Q followed by GC-MS analysis showed 2,5-dimethyl-3-(2-methylbutyl)pyrazine as the major volatile released by *W. auropunctata* workers while 3-methyl-2-(2-methylbutyl)pyrazine was only detected in trace amounts. In laboratory bioassays, *W. auropunctata* workers were attracted and arrested by both pyrazines. Field bioassays with *Wasmannia auropunctata* (Roger) show that the pheromone components both attract and arrest ants in a natural environment. A dose response assay with 2,5-dimethyl-3-(2-methylbutyl)pyrazine showed maximal ant response to a 1 mg pheromone lure, a dose which remained attractive for 8 days under field conditions. Several of the field experiments included peanut butter baits, a lure currently used for detection. However, ant counts at peanut butter baits were not greater than at controls suggesting that peanut butter does not produce volatiles that attract ants. With the aim of developing

management applications, a series of bioassays were conducted with 2,5-dimethyl-3-(2-methylbutyl)pyrazine in combination with food baits. A separate assay was conducted with Tanglefoot, a sticky catch material. In feeding bioassays, the pheromone decreased consumption of peanut butter and solutions of protein and sugar. Tanglefoot squares failed to catch *W. auropunctata* with any of the lures tested. The field responses of *W. auropunctata* to alarm pheromone lures show a mixed potential for control applications. While the strong attraction and longevity of lures is promising, the inability to increase bait consumption or capture ants with Tanglefoot presents obstacles to using these alarm pheromone components for ant management.

Completion of tasks 1-3, as outlined in the scope of services and restated below, are outlined in the following text. Submission of this report completes task 4. Activities to disseminate the results of this research are outlined at the end of this report.

1. Semiochemicals in currently utilized food-based attractants will be identified using gas chromatograph-electroantennogram (GC-EAD) techniques for volatile chemicals and/or high pressure liquid chromatographic techniques for non-volatile chemicals. The approach will be to identify semiochemicals in various food attractants such as peanut butter and other food baits used in current survey and detection methods. We will also investigate the possibility that LFA possesses trail following or alarm pheromones which may be able to increase trap attractiveness (Greenberg 2000, Hughes 2001 & 2002).

2. Individual or groups of separated chemicals will be identified and assayed on laboratory and wild populations of LFA for attraction, as arrestants and their role as "signaling molecules" for other identified behaviors that might be useful in future control strategies such as mating or trail disruption, vector loading and intracolony recognition factors.

3. In addition to food attractants we also propose to look at ant-specific semiochemicals as behavior-modifying agents. This will be accomplished by performing surface washes of various identified colonies to see if colony-specific cuticular hydrocarbons exist in this species. Hexane washes of ants will be used in ant bioassays and if successful individual compounds separated and identified using GC-MS. These molecules will also be assayed for behavioral activity in the lab and if appropriate in the field.

Methodology:

Insects and Field Location

Wasmannia auropunctata workers and alates were collected from a shade house at the Agricultural Farm Laboratory of the University of Hawaii at Hilo (GPS coordinates: 19.650668, -155.050505) and shipped overnight in 25 ml vials to Eastern Mennonite University, Harrisonburg, VA. Ants were housed in 1.2 L plastic containers (13 cm × 13 cm × 7 cm high), which contained the vial in which the ants were shipped covered in foil to provide a retreat, a foraging area containing minced insects, and 4 ml cotton-topped vials of water and a 50% honey solution. Two colony containers were housed in a larger 7.6 L outer container to prevent escape. All containers were vented with mesh (7 cm^2 per lid). Insect-a-Slip (Fluon, PTFE-30 BioQuip Products, Inc., Rancho Dominguez, CA) was applied to the sides of all containers to prevent escape. Ants were kept at ambient temperature (near 24 °C) on a 16:8 hour light/dark cycle.

All field tests were conducted in a macadamia nut orchard on the island of Hawaii, Papaikou, HI (GPS coordinates: 19.787029, -155.124443), from 12 May 2009 to 26 May 2009. Tests were performed in macadamia nut trees where *W. auropunctata* trails were readily observed. The highest numbers of ants were found in heavily moss-covered trees, with higher activity periods coinciding with cooler temperatures. Average daily temperatures varied from 22-26 °C and relative humidity from 65-81 %.

Extraction and headspace sampling

Wasmannia auropunctata workers (approx. 200) were frozen at -70 °C and extracted for 5 min in CH_2Cl_2. Trisected ants (heads, thoraxes, and gasters) were also separately extracted in

the same manner. Extracts were transferred into conical glass tubes, concentrated under a purified nitrogen stream, and refrigerated until analysis by GC-MS.

Headspace samples were collected using a solid phase microextraction (SPME) fiber coated with polydimethylsiloxane (PDMS; film thickness 100μm: Supleco Inc., Bellefonte, PA) with both live and crushed ants. Live workers (approx. 100) were transferred to a clean glass container and allowed to settle for 15 min. The SPME fiber was inserted through the lid of the container and exposed for 15 min. Collections from crushed ants were conducted as described by Di Tullio *et al.* (2003). Whole ants, separated head or gasters (5-10 per extract) were placed at the bottom of glass melting point tubes (FP-1, Mettler-Toledo Inc., Columbus, OH) cut to be 50 mm long. Samples were crushed with a wire before the fiber was inserted in the tube, which was then sealed with Teflon tape. Extractions were performed at 100 °C for 30 min.

Headspace sampling was also performed with Porapak Q (50-80 mesh bulk packing material, Supleco). Absorbent (1 g) was packed between glass wool plugs in a Pasteur pipette, washed with CH_2Cl_2, and dried with purified nitrogen. Purified air was pumped through a glass tube containing workers (approx. 500) for 50 min, with volatiles collecting on the Porapak Q column. Absorbent was washed with CH_2Cl_2 (1 ml), which was concentrated under a purified nitrogen stream and refrigerated until analysis by GC-MS.

Gas chromatography-mass spectrometry

Ant extracts, headspace collections, and synthetic pyrazines were analyzed by gas chromatography-mass spectrometry (GC-MS) using two instruments. The first, located in Hawaii, consisted of an Agilent Technologies 6890N gas chromatograph interfaced to a Hewlett-Packard 5973 Mass Selective Detector equipped with HP-5MS column (30 m × 0.25 mm ID,

0.25 μm film thickness). The standard temperature program used was 80 °C to 220 °C at 10 °C/min with a 1 min start delay. The injector temperature was set at 250 °C and helium was used as a carrier gas (1.1 ml/min). The second instrument, located in Virginia, was a Hewlett Packard G1800A GCD system equipped with one of the following columns: ZB-5 (30 m × 0.25 mm ID, 0.25 μm film thickness); Rtx-1 (60 m × 0.32 mm ID, 1.0 μm film thickness); DB-225MS (30 m × 0.25 mm ID, 0.25 μm film thickness); Rt-BDEXm (30 m × 0.25 mm ID, 0.25 μm film thickness). The temperature program used was 60 °C to 250 °C at 10 °C/min with a 1 min start delay. The injector temperature set at 250 °C and helium was used as a carrier gas (1.0 ml/min). Mass spectral data were analyzed using a NIST 98 mass spectral database.

Nuclear Magnetic Resonance and Infrared Spectroscopy

^{1}H NMR spectra were obtained with a Bruker DRX-400 FT-NMR spectrometer equipped with a broadband gradient probe. All spectra were recorded in deuterated chloroform with 1% TMS as an internal standard. Fourier transform IR (FT-IR) spectra were collected with a Thermo Nicolet Magna-IR 560 Spectrometer. Liquid samples were analyzed using KBr plates.

Synthesis

Pyrazines of interest were synthesized by an alkyl-zinc cross-coupling reaction modified from Sato and Matsuura (1996) (Figure 1). All solvents and reagent compounds were purchased from Sigma-Aldrich, Inc., Saint Louis, MO or Fisher Scientific, Rochester, NY unless otherwise noted. The alkyl-zinc reagents were prepared in two steps from either 1-bromo-3-methylbutane (Sigma-Aldrich) or racemic (+/–)-1-bromo-2-methylbutane (Frinton Laboratories, Inc., Vineland, NJ) via a Grignard reaction followed by addition of zinc bromide. These alkyl-zinc

reagents were added to either 3-chloro-2,5-dimethylpyrazine (Sigma-Aldrich), 2-chloro-3-methylpyrazine (Pyrazine Specialties, Inc. Atlanta, GA) or a mixture of isomers 2-chloro-(3),(5),(6)-methylpyrazine (Pyrazine Specialties, Inc. Atlanta, GA), in the presence of the [1,2-bis(diphenylphosphino)-propane] dichloronickel catalyst in MeTHF at rt. After 2.5 hours the reaction was quenched with ice water, filtered, and extracted. The compounds were purified by flash chromatography to yield a viscous yellow liquid for all products: 2,5-dimethyl-3-(3-methylbutyl)pyrazine (3-MeBu-diMePy), GC/MS (EI, 70 eV) m/z (relative intensity) 177 $[M-1]^+$ (1), 163 (8), 149 (1), 135 (14), 122 (100), 107 (3), 80 (4), 53 (7); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.990 (6H, d), 1.557 (2H, m), 1.690 (1H, m), 2.498 (3H, s), 2.537 (1H, s), 2.778 (3H, m), 8.158 (1H, s). Racemic (+/–)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (2-MeBu-diMePy), (95% purity – including both enantiomers); GC/MS (EI, 70 eV) m/z (relative intensity) 177 $[M-1]^+$ (1), 163 (6), 149 (4), 135 (2), 122 (100), 107 (2), 80 (4), 53 (8); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (3H, d), 0.94 (3H, t), 1.27 (2H, m). 1.44 (1H, m), 2.50 (3H, s), 2.53 (3H, s), 2.80 (2H, dd), 8.16 (1H, s); IR wavenumber, cm^{-1} (percent transmittance) 3041 (66), 2960 (49), 2927 (51), 2874 (56), 2856 (59), 1451 (55), 1374 (58), 1168 (65), 1076 (68). Racemic (+/–)-3-methyl-2-(2-methylbutyl)pyrazine (2-MeBu-MePy), (98% purity – including both enantiomers); GC/MS (EI, 70 eV) m/z (relative intensity) 163 $[M-1]^+$ (1), 149 (6), 135 (5), 121 (2), 108 (8), 93 (3), 67 (7), 53 (5); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.91 (3H, d), 0.95 (3H, t), 1.27 (2H, m), 1.44 (1H, m), 2.58 (3H, s), 2.85 (2H, dd), 8.29 (1H, s), 8.34 (1H, s); IR wavenumber, cm^{-1} (percent transmittance) 3045 (70), 2961 (18), 2928 (27), 2875 (37), 1234 (80), 1460 (39), 1405 (22), 1378 (50), 1168 (46).

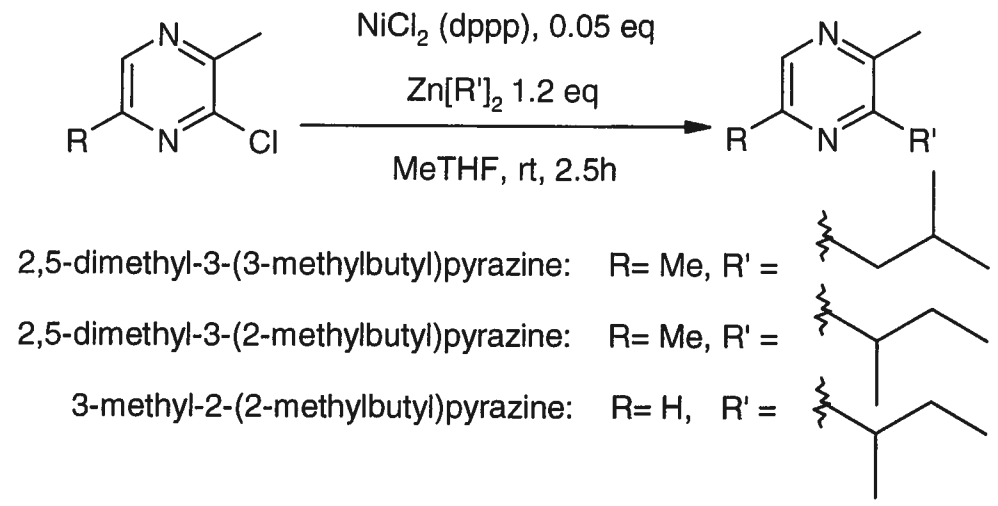

Figure 1. Scheme modified from Sato and Matsuura (1996) for the synthesis of alkylpyrazines.

Pheromone Bioassays

Bioassays used to measure activity, attraction, and arrestment of *W. auropunctata* in response to synthetic pheromone components were modified from Fujiwara-Tsujii *et al.* (2006), Howard *et al.* (1982), and Vander Meer *et al.* (1988). Racemic 2-MeBu-diMePy and 2-MeBu-MePy, as prepared above, were used in all bioassays.

Experiments 1 and 2. The first set of bioassays accessed *W. auropunctata* responses to the synthetic pyrazines either singly or in blends. These assays were conducted in Petri dishes (85 mm ID x 45 mm high) with 25 mm diameter circles drawn on the underside of each dish. Ten ants were placed in each dish and left undisturbed for 2 minutes. Each test was initiated by placing a treated filter paper disk (5 mm diameter) at the center of the marked circle. Total number of ants crossing (into or out of) the center circle was recorded over 5 minutes. Arrestments, the number of ants in contact with the disk, were recorded every minute for 5 minutes. An ant from the colony being tested was crushed between filter paper disks and served

as a positive control. The negative control was a filter paper disk treated with CH_2Cl_2. Petri dishes were cleaned with ethanol (95%) between replicates.

Experiment 1 was conducted to assess the relative activity and synergy of the synthetic alkylpyrazines. Three treatments were assayed in the manner described above: 200 ng of 2-MeBu-diMePy, 200 ng of 2-MeBu-MePy, and 200 ng of both pyrazines (all applied in CH_2Cl_2). The 200 ng dose was chosen to reflect the maximum amount of alkylpyrazine pheromone present in individual ants as estimated by Howard *et al.* (1982).

Experiment 2 was conducted to assess ant responses to varying concentrations of synthetic pyrazines. GC analysis of ant extracts showed 2-MeBu-diMePy/2-MeBu-MePy ratios ranging from 4.5:1 to >100:1, with the most common ratio being in the 100:1 range. Therefore, the following treatments of 100:1 2-MeBu-diMePy/2-MeBu-MePy were used: 200 ng:2 ng, 20 ng:200 pg, and 2 ng:20 pg. Additionally a 2 µg:200 ng (10:1) treatment was tested.

Experiments 3 and 4. A second type of bioassay was conducted by placing treated filter paper on a small inverted watch-glass (25 mm diameter, 3 mm center height, 1 mm thickness/height at edge) within the foraging area of the colony containers. This approach eliminated the disruption caused by transferring ants, and the height of the watch-glass provided an obstacle to decrease random foraging in the recording area. Three treatments were assayed: 200 ng of 2-MeBu-diMePy; 200 ng of 2-MeBu-MePy; and 200 ng of both pyrazines (all applied in CH_2Cl_2). Each treatment was run in every colony to control for differences in colony size, health, and activity. Arrestments were recorded as in Experiments 1 and 2. Crossings were recorded as onto or off of the watch-glass each minute for 5 minutes.

In Experiment 3, an ant from the colony being tested was crushed between filter paper disks and served as a positive control. The negative control was a filter paper disk treated with

CH_2Cl_2. In Experiment 4, a separated ant head from the colony being tested, rather than a whole ant, was crushed between filter paper disks and served as a positive control. The negative control was a filter paper disk treated with CH_2Cl_2.

Experiment 5. This bioassay was conducted in order to determine the particular body segment of a crushed ant that produced the observed attractive effect. Ants were trisected into head, thorax, and gaster. Each of these sections was crushed between filter paper and introduced on a watch-glass to the foraging area as in Experiments 3 and 4. Crossings and arrestments were again recorded as in Experiments 3 and 4.

Experiments 6-8. Four map pins were used to define a 4 x 4 cm square counting area located 2 cm from an observed *W. auropunctata* trail. A treated rubber septum (13 mm snap-on stopper rubber septa, Wheaton, Millville, NJ) was pinned to the center of each counting area. Counting areas were limited to one per tree and remained fixed throughout an experiment. Treatments were rotated so that each tree/ant trail was exposed to every treatment. This helped control for variation in ant numbers and activity. Following attractive pheromone treatments, residual ant activity was sometimes observed in the counting area. In these cases, more rest time was allowed or ants were blown off the counting area between replicates. Despite these measures, some residual ant activity due to previous treatments is suspected.

Experiment 6 assessed the concentration at which the pheromone is most active. 2-MeBu-diMePy lures were prepared on rubber septa at 100 ng, 10 µg, and 1 mg doses and compared to a CH_2Cl_2 control (120 replicates). Ants within the marked areas were counted at 5-min. intervals for 30 min.

Experiment 7 examined the relative attractancy of both *W. auropunctata* pheromone pyrazines. Four treatments were assayed: 1 mg 2-MeBu-diMePy, 1 mg 2-MeBu-MePy, and

blends of 1 mg : 100 µg and 1 mg : 10 µg of 2-MeBu-diMePy to 2-MeBu-MePy. The ratios reflect concentrations found in ant extracts (Showalter and others 2009a). Initially, ant counts were made every 5 min. for 30 min., as in Experiments 1 and 3. However, on the day of this experiment most test areas were overrun by hundreds of ants within a few minutes of beginning a trial, making accurate ant counts extremely difficult. To address this problem, the time to attract a given number of ants (10, 20, and 30 ants) was recorded to assess initial attraction (16 replicates for each number of ants). If 30 ants were not attracted within 10 min., only the maximum number of ants present during the 10 min. was recorded. The time to attract ants in these cases was assigned as 10 min. for purposes of analysis because insufficient ants were attracted for an exact time recording.

Experiment 8 measured the attractancy of 2-MeBu-diMePy against peanut butter (Safeway Brand creamy), a widely used survey tool. Rubber septa, treated with 1 mg 2-MeBu-diMePy (60 replicates), filled with peanut butter (55 replicates), or treated with CH_2Cl_2 alone (60 replicates), were pinned to the center of the marked area. Ants within the marked area were counted at 5-min. intervals for 30 min.

Experiment 9. This experiment was designed to assess the ant population monitoring potential of 2-MeBu-diMePy in combination with a sticky catch material (Tanglefoot). A thin layer of Tanglefoot (The Tanglefoot Company, Grand Rapids, MI) was applied in ~5 x 5 cm squares on trees, 2-3 cm from ant trails. Rubber septa treated with 1 mg 2-MeBu-diMePy, peanut butter, or CH_2Cl_2 controls were placed in the center of the Tanglefoot square (8 replicates). Ants were attracted by some lures but were not caught in the Tanglefoot. Therefore, the number of ants within 1 cm of the Tanglefoot square perimeter was observed and recorded. Counts were taken at intervals over 8 days.

*Experiment 10.*This experiment assessed the impact of 2-MeBu-diMePy on feeding behavior. Experiments 10.1-10.3 used feeding vials, as adapted from Greenberg and Klotz (2000). The feeding apparatus consisted of a 1.5 ml glass vial (12 x 32 mm clear glass crimp top vial, Supelco Inc. Bellafonte, PA) with a 3 x 3 cm square of conically perforated membrane (Weed Block, Easy Gardener Products, Inc., Waco, TX) placed between the previously filled vial and crimp lid (13 mm aluminum crimp seal without septum, Supelco Inc. Bellafonte, PA). Vials were filled with solutions of 2 g hydrolyzed protein/40 ml H_20 or 10 g sucrose/40 ml H_20.

We tried multiple methods of attaching the vials to the trees. The most reliable technique was Velcro in combination with epoxy adhesive. The conjoining sides of the Velcro were separated, and one half was epoxied to a chosen location in a tree, while the corresponding half was epoxied to the glass vial. This method allows for optimal vial placement (sheltered and vertical) and ease of setup and vial recovery.

To account for the different evaporation rates of sugar and protein solutions, evaporation controls were also set out in the field. These consisted of the same basic feeding vial apparatus, placed in a plastic container with a mesh lid. This excluded the ants, but also exposed the evaporation controls to the same temperature and humidity as the treatments. The data from the evaporation controls were subtracted from the differences in feeding vial weights to calculate the actual liquid consumed.

A preliminary trial, Experiment 10.1, was conducted to determine if these *W. auropunctata* populations displayed a preference for sugar or protein food sources. Feeding behavior was determined by measuring consumption of three liquid food sources: a sugar solution (10 g sugar/40 ml H_20), a protein solution (2 g protein/40 ml H_20), and a sugar/protein solution (an equal combination of the two). Experiments 10.2 and 10.3 assessed the impact of 2-

MeBu-diMePy on the consumption of protein and sugar solutions, respectively. Initially, pheromone was applied directly to the membrane of the feeding vial. However, in subsequent trials rubber septa treated with the pheromone were glued to the vials. Both treatments received 1 mg 2-MeBu-diMePy and were compared to a CH_2Cl_2 treated control. The vials were weighed before and after field exposure to *W. auropunctata*.

Experiment 10.4 assessed the impact of 2-MeBu-diMePy on ant consumption of peanut butter. Rubber septa treated with 1 mg 2-MeBu-diMePy or CH_2Cl_2 controls were hot-glued onto peanut butter-filled plastic caps (6 replicates). These were attached to the trees in the same epoxy/Velcro method as Experiment 10.1-10.3. Peanut butter-filled caps were weighed before and after field exposure.

Analysis

Results of bioassays were analyzed using ANOVA followed by Tukey's HSD test (alpha = 0.05) to compare means. Arrestment data were normalized by log transformation before analysis. All analyses of significance were made at the $P < 0.05$ level. Field data were analyzed using ANOVA followed by Tukey's HSD test (alpha = 0.05), with the exceptions of data from Experiments 10.2-10.4, in which *t*-Tests were performed.

Results and Discussion:

Alkylpyrazines were found to constitute the major volatiles released from peanut butter when assayed by head-space using solid phase microextraction (SPME) and analysis by gas-chromatography/mass-spectrometry (GC/MS). Field testing of these compounds showed little or no attraction. Likewise, laboratory bioassays (Figure 2) did not show strong attraction to peanut

butter over Spam baits and suggest that ant foraging behavior not volatile attraction are responsible for bait discovery.

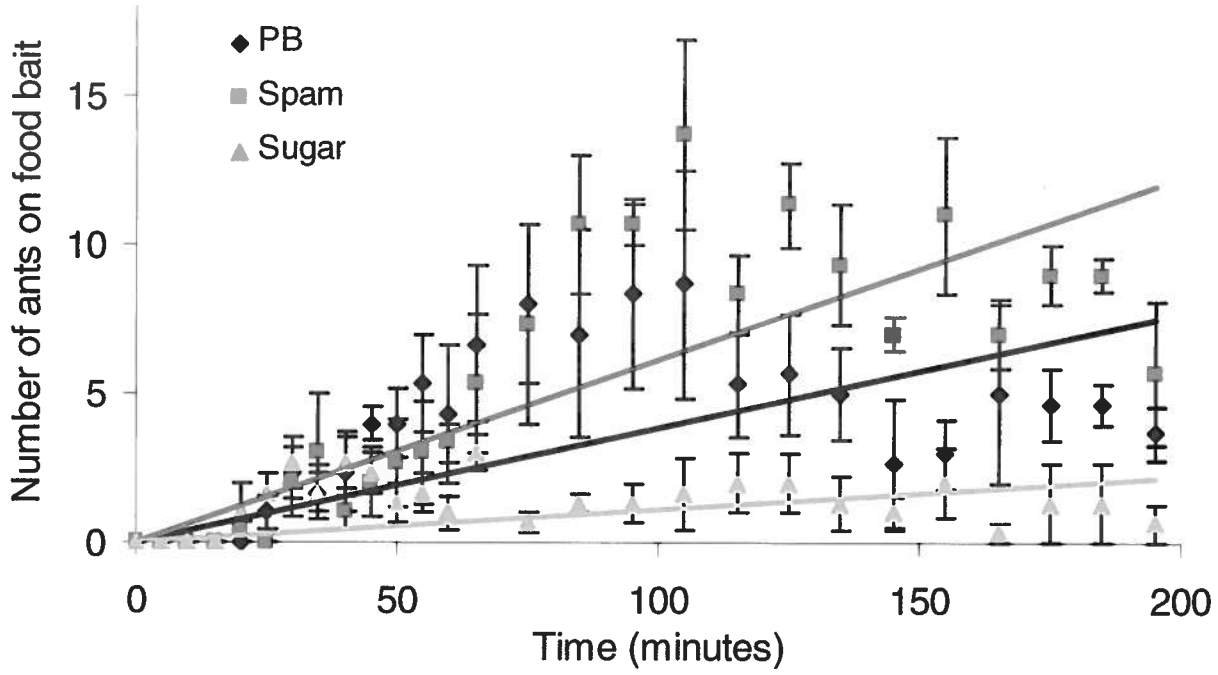

Figure 2. Ant discovery of different food baits in a laboratory bioassay.

Extraction and Headspace Sampling

Gas chromatograms of ant extracts revealed a trisubstituted and a disubstituted pyrazine, in ratios ranging from 4.5:1 to greater than 100:1 respectively. Extracts from separated ant heads, thoraxes, and gasters confirmed the trisubstituted pyrazine to be the main volatile component in the head, as found by Howard et al. (1982). Other volatiles, including a number of sesquiterpenoids, were present in the gaster. Headspace analyses with SPME and Porapak Q also showed 2-MeBu-diMePy to be the major released volatile component from both live and crushed ants. 2-MeBu-diMePy constituted >98 % of the detected volatiles in SPME collections of

separated ants heads. Small amounts of 2-MeBu-MePy were also detected by both headspace analysis techniques.

Compound Identifications

3-MeBu-diMePy was prepared according to methods described by Sato and Matsuura (1996), and its structure was confirmed by NMR and MS. Coinjection of 3-MeBu-diMePy with an extract of *W. auropunctata* on GC produced two distinct chromatographic peaks, indicating that the trisubstituted ant pyrazine was not 3-MeBu-diMePy. Comparison of the trisubstituted ant pyrazine mass spectrum with database mass spectra suggested the structure 2-MeBu-diMePy (Figure 3). Key differences in the mass spectra of 3-MeBu-diMePy and 2-MeBu-diMePy occur with the cleavage of the alkyl side chain between C-2 and C-3, which are reflected in the relative intensities of the fragmentation ion peaks, M-29 (*m/z* 149) and M-43 (*m/z* 135) (Figure 3). Cleavage at this position yields a prominent M-29 peak and a reduced M-43 for 2-MeBu-diMePy while the reverse is seen with 3-MeBu-diMePy. This identification was confirmed by the synthesis of 2-MeBu-diMePy and its sequent retention time comparison to the trisubstituted ant pyrazine on several GC columns. Coinjection of 2-MeBu-diMePy with an extract of *W. auropunctata* on GC produced no additional chromatographic peak.

Mass spectrometry analysis suggested the disubstituted ant pyrazine to be a single methyl analog of 2-MeBu-diMePy (Figure 3), of which three regioisomers are possible. A mixture of these isomers was synthesized from their corresponding chloropyrazine mixture. Isomer identifications were assigned based on elution order: 5-, 6-, and 3-methyl-2-(2-methylbutyl)pyrazine, as per Hwang (1993) and Hwang (1995) on a similar nonpolar column. Retention time comparison suggested 2-MeBu-MePy as the isomer present in ant extracts. This

was confirmed by synthesis of pure 2-MeBu-MePy and its sequent retention time comparison to the disubstituted ant pyrazine on several GC columns. Coinjection of 2-MeBu-MePy with an extract of *W. auropunctata* on GC produced no additional chromatographic peak.

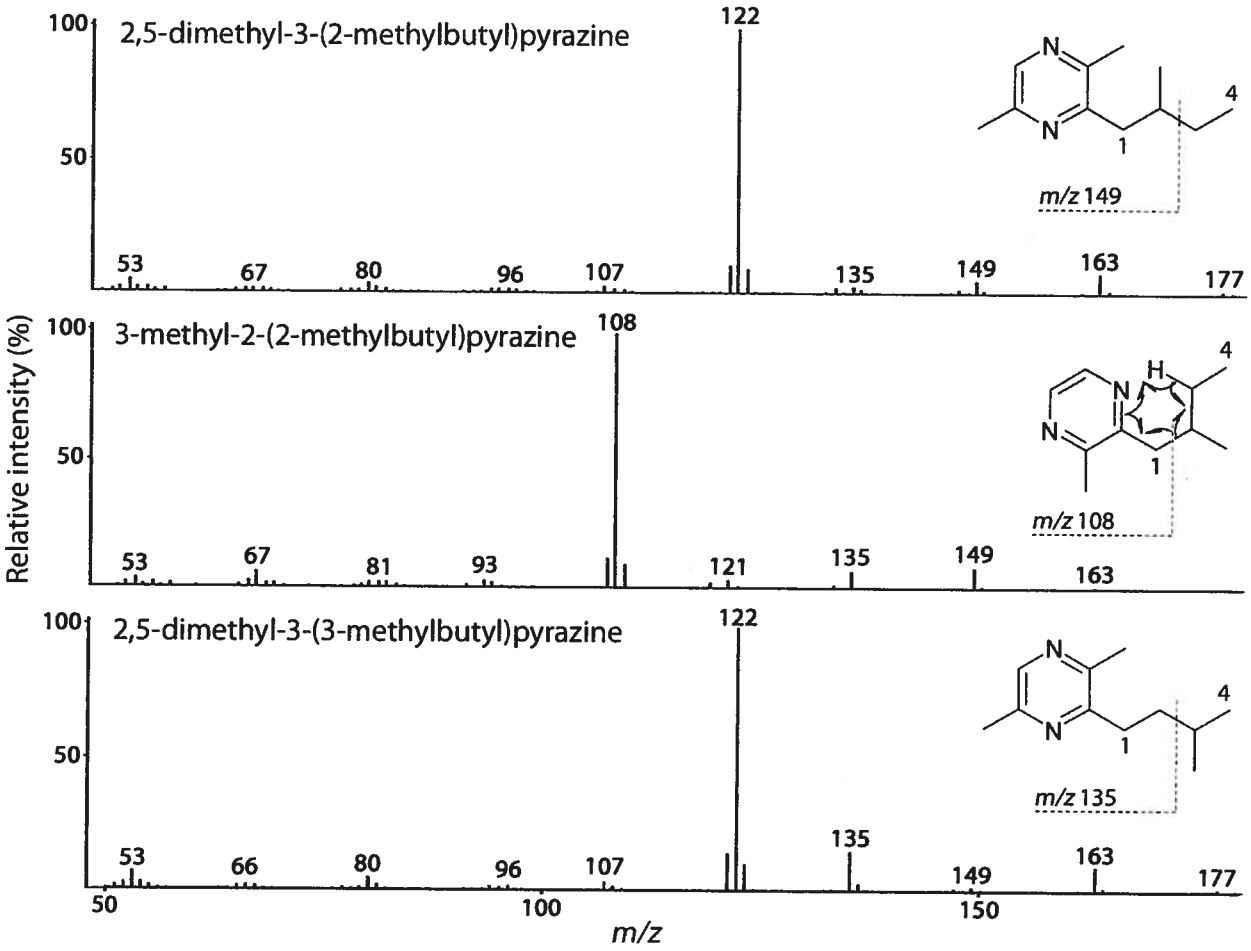

Figure 3. Electron impact mass spectra (EIMS; 70 eV) of alkylpyrazines from CH_2Cl_2 extracts of *W. auropunctata*. Numbering system indicated for alkyl side chain. 2-MeBu-diMePy and 3-MeBu-diMePy alkyl side chain fragmentation between C-2 and C-3 are indicated. 2-MeBu-MePy shows a base peak at *m/z* 108, consistent with the formation of the resonance stabilized fragment indicated, and is in good agreement with the mass spec data given by Friedel et al. (1971).

Both 2-MeBu-diMePy and 2-MeBu-MePy are chiral and an attempt to assign the absolute configurations of the alkylpyrazines present in *W. auropunctata* was made using a chiral GC column (Rt-BDEXm). Synthetic racemic 2-MeBu-diMePy and 2-MeBu-MePy, as well as *W. auropunctata* extracts, were analyzed. Although multiple temperature profiles, sampling and injection techniques were tried, no enantiomeric separation were achieved, thus we were unable to assign the absolute configurations of either natural alkylpyrazine.

Bioassays

In Experiment 1, ant responses to synthetic alkylpyrazines at a 200 ng dose, the maximum amount of alkylpyrazine pheromone present in individual ants as estimated by Howard et al. (1982), were tested (Figure 4). Arrestments of ants by whole crushed ant, 2-MeBu-MePy, and the combination of both pyrazines were not significantly different, but these treatments all arrested more ants than 2-MeBu-diMePy or the negative control (ANOVA: $F = 9.60$, df = 4, $P < 0.001$). The number of crossings induced by 2-MeBu-MePy and by the combination of both pyrazines as significantly greater than the negative control (ANOVA: $F = 5.79$, df = 4, $P = 0.002$). Behaviors such as biting, carrying, or dragging the filter paper disks to another part of the arena were observed with the crushed ant and occasionally with synthetic pyrazines, but never with the negative control.

Experiment 2 did not show a significant dose response for the range tested (2 µg/200 ng to 2 ng/20 pg). The synthetic pyrazine dilution series had significantly fewer arrestments than the crushed ant positive control for all concentrations tested (ANOVA: $F = 12.508$, df = 5, $P < 0.001$), while crossings did not significantly differ (ANOVA: $F = 1.619$, df = 5, $P = 0.185$).

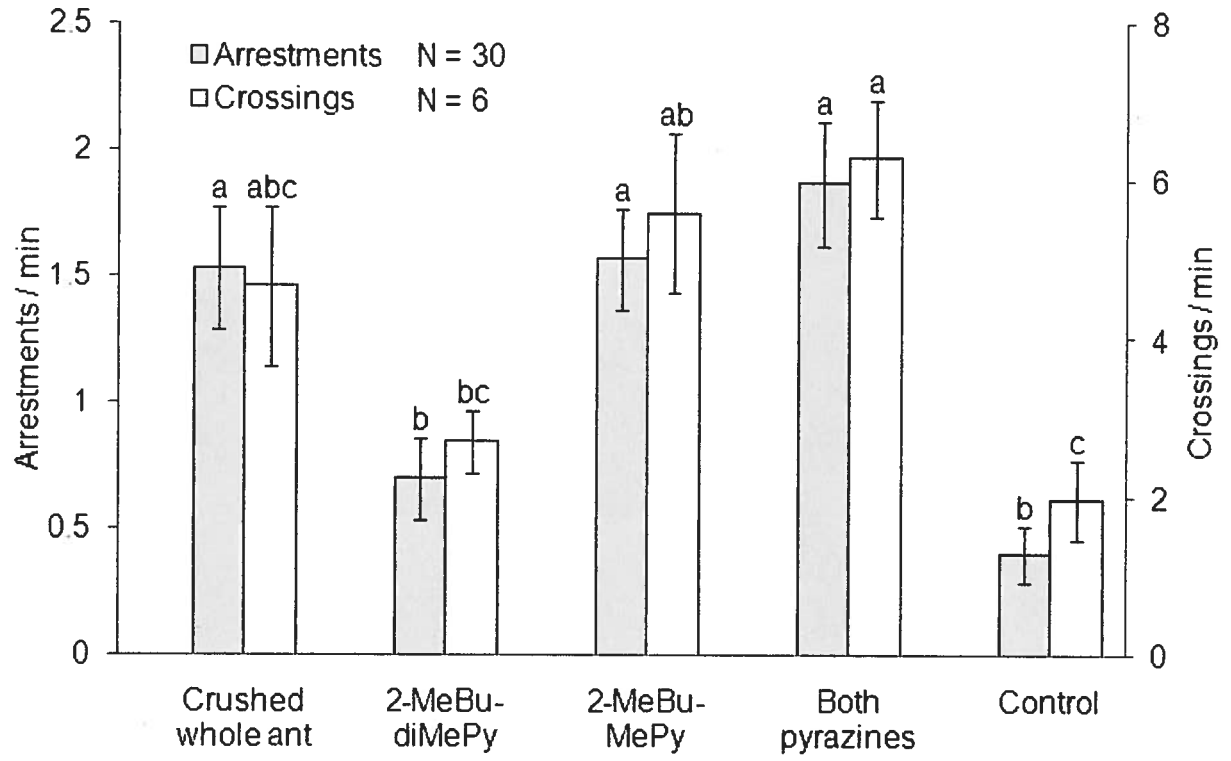

Figure 4. Numbers (mean ± SE) of *W. auropunctata* crossing circle perimeter and arresting on filter paper treatments in Experiment 1. Synthetic components were tested alone and at a 1:1 ratio combination. Letters represent significant differences ($P < 0.05$) between arrestments and crossings of different treatments (ANOVA, followed by Tukey's HSD).

Bioassays conducted in the foraging area within colonies produced data that conflicted with the previous experiment regarding the most active pyrazine (Figure 5). In Experiment 3, arrestments were not significantly different between 2-MeBu-diMePy and whole crushed ant treatments, while both were significantly different from the negative control (ANOVA: $F = 12.21$, df = 4, $P < 0.001$). Synthetic pyrazines did not produce significantly different numbers of crossings compared with the negative control (ANOVA: $F = 4.17$, df = 4, $P = 0.003$).

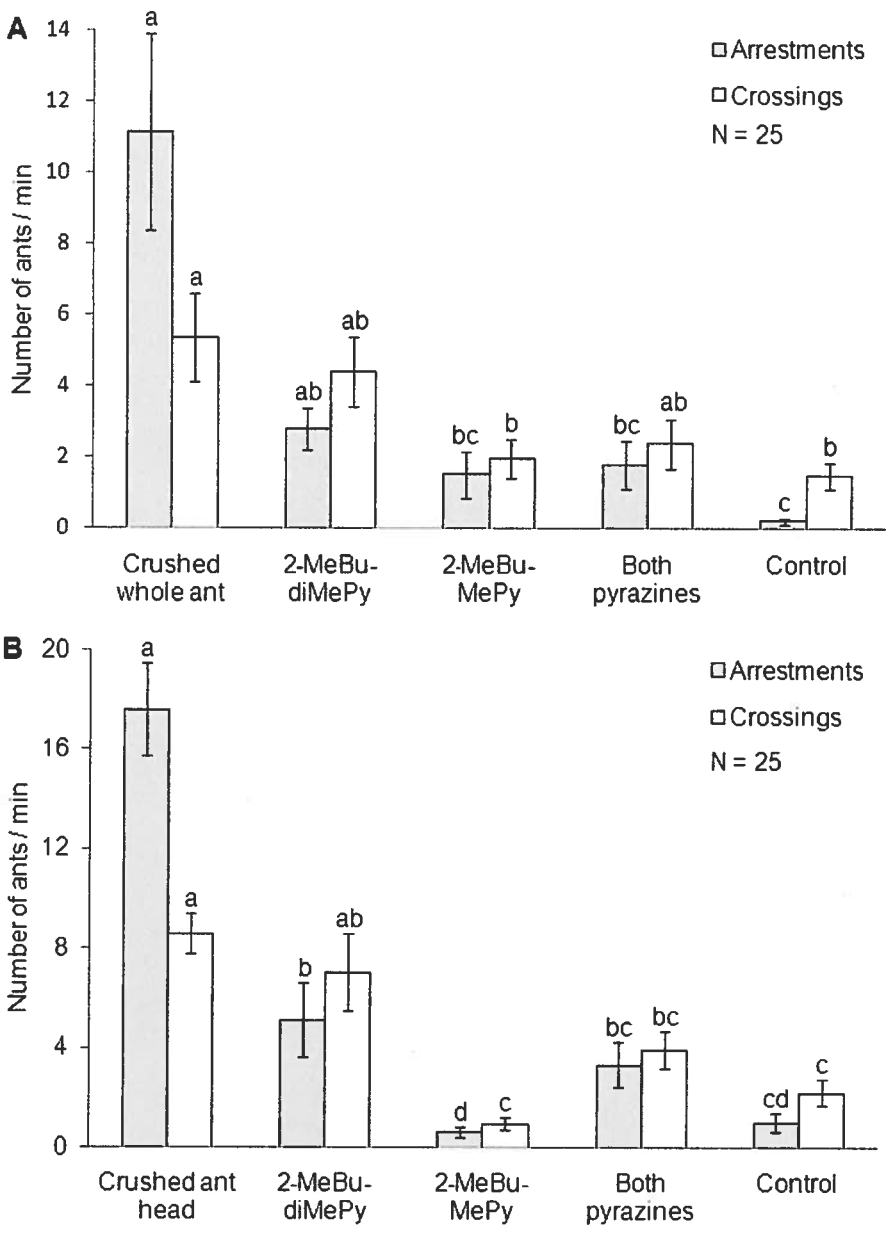

Figure 5. Numbers (mean ± SE) of *W. auropunctata* crossing inverted watch-glass and arresting on filter paper treatment in Experiments 3 and 4. (A) In Experiment 3, positive control is one whole ant crushed between two filter paper disks. (B) In Experiment 4, positive control is one ant head crushed between two filter paper disks. Within each experiment, letters indicate significant differences ($P < 0.05$) between arrestments and crossings of different treatments (ANOVA, followed by Tukey's HSD).

In Experiment 4, however, arrestments in the 2-MeBu-diMePy treatment were significantly lower than in the crushed ant head treatment, but still higher than in the 2-MeBu-MePy treatment and in the control (ANOVA: $F = 34.53$, df = 4, $P < 0.001$). Crossings in the 2-MeBu-diMePy treatment were not significantly different from the ant head treatment, but they were significantly higher than in the 2-MeBu-MePy treatment and in the control (ANOVA: $F = 13.36$, df = 4, $P < 0.001$). Treatments of combined pyrazines did not increase attraction in either Experiment 3 or 4.

Experiment 5 compared ant responses when exposed to trisected ant body parts (Figure 6). The crushed head caused significantly more arrestments than either the crushed thorax or gaster (ANOVA: $F = 36.67$, df = 2, $P < 0.001$). The crushed head induced significantly more crossings than the crushed thorax (ANOVA: $F = 4.38$, df = 2, $P < 0.019$).

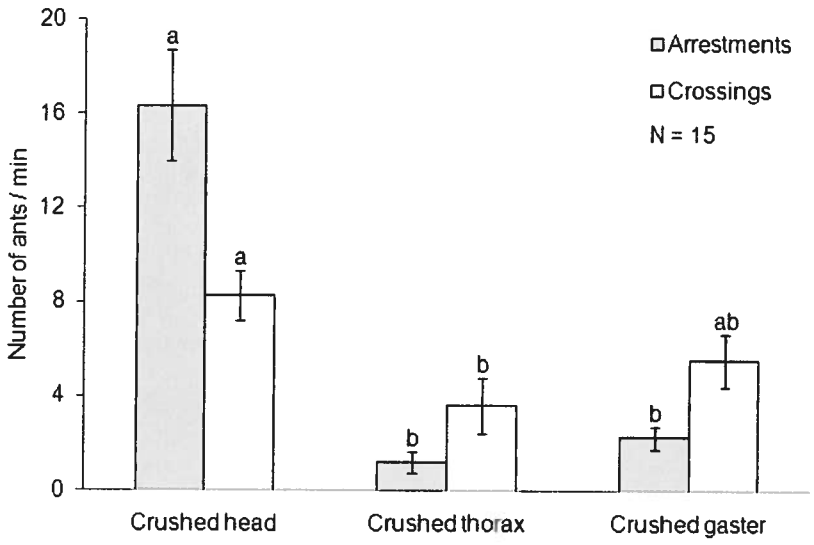

Figure 6. Numbers (mean ± SE) of *W. auropunctata* crossing inverted watch-glass and arresting on filter paper treatment. Experiment 5 tested attractiveness of the three ant segments. Letters represent significant differences ($P < 0.05$) between arrestments and crossings of different treatments (ANOVA, followed by Tukey's HSD).

In Experiment 6, the most active pheromone concentration was determined (Figure 7). The number of ants observed in counting areas was greatest with 1 mg lures, while only the 100 ng dose was not significantly greater than the control (ANOVA: $F = 111.044$, df = 3, $P < 0.001$). When an attractive lure was placed in a counting area, *W. auropunctata* workers generally began to respond by orienting toward the pheromone source within a few minutes of placement. 2-MeBu-diMePy lures appeared to increase ant locomotion compared to the normal pace of ants travelling along trails. Ant distribution within the counting area was non-random, with many more ants aggregating on or in close proximity to the pheromone-treated septa.

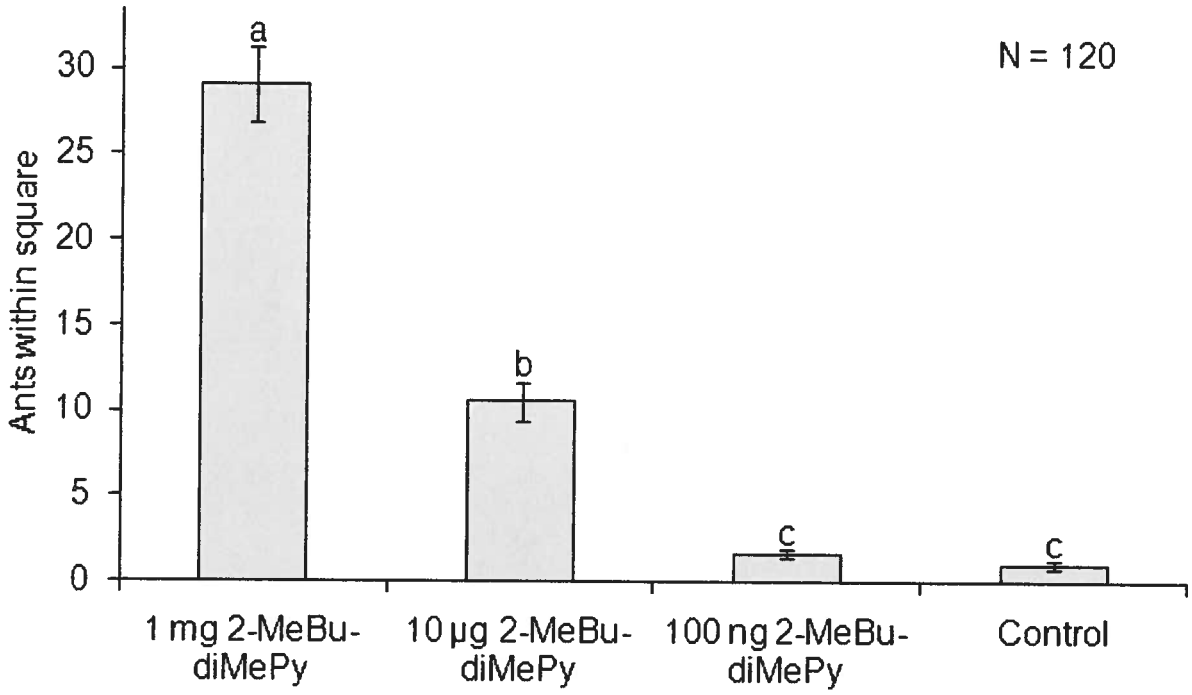

Figure 7. Experiment 1, numbers (mean ± SE) of *W. auropunctata* counted in defined area for each treatment at 5-minute intervals. Letters represent significant differences ($P < 0.05$) between treatments (ANOVA, followed by Tukey's HSD).

Experiment 7 measured the relative attractancy of the two *W. auropunctata* mandibular pyrazines (Table 1). There were no significant differences between pyrazine treatments for the time to 10 ants (ANOVA: $F = 0.115$, df = 2, $P = 0.951$), 20 ants (ANOVA: $F = 0.095$, df = 2, $P = 0.963$), or 30 ants (ANOVA: $F = 0.123$, df = 2, $P = 0.946$).

Table 1. Experiment 2, relative attractiveness of *W. auropunctata* mandibular pyrazines.

	Time in seconds (mean ± SE)* for a given # of ants to be observed in recording area			
	2-MeBu-diMePy	2-MeBu-MePy	2-MeBu-diMePy/ 2-MeBu-MePy (10:1)	2-MeBu-diMePy/ 2-MeBu-MePy (100:1)
10 ants	219 ± 49 a	206 ± 53 a	209 ± 49 a	245 ± 56 a
20 ants	350 ± 59 a	390 ± 52 a	368 ± 54 a	375 ± 52 a
30 ants	427 ± 49 a	461 ± 46 a	423 ± 51 a	434 ± 50 a

*Means within a row followed by the same letter are not significantly different ($P > 0.05$) using Tukey's HSD test.

In Experiment 8, pheromone attractancy was measured against peanut butter (Figure 8). More ants were observed with the 1 mg 2-MeBu-diMePy lure than either the peanut butter or control, which were not significantly different from each other (ANOVA: $F = 154.315$, df = 2, $P < 0.001$).

Experiment 9 attempted to assess the ant population monitoring potential of 2-MeBu-diMePy in combination with Tanglefoot, but was converted into a longevity study of pheromone attractancy in comparison with peanut butter attractancy (Figure 9). The pheromone was found to have significantly higher attractancy at every time interval, but no significant difference was found between peanut butter and control. In contrast to the short-term observations of Experiments 1-3, in which ant locomotion seemed to increase, *W. auropunctata* surrounding pheromone-treated lures in Experiment 4 were largely quiescent.

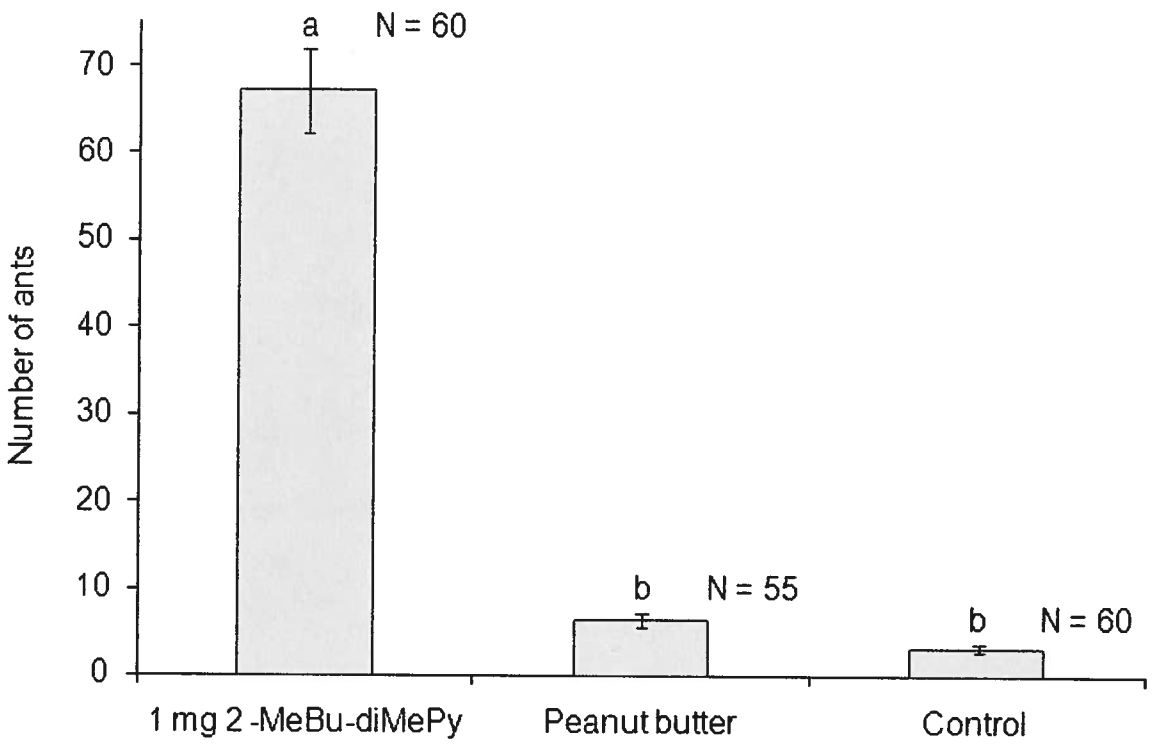

Figure 8. Experiment 3, numbers (mean ± SE) of *W. auropunctata* counted in defined area for each treatment at 5-minute intervals. Letters represent significant differences ($P < 0.05$) between treatments (ANOVA, followed by Tukey's HSD).

Experiment 10 consisted of a series of trials designed to measure the impact of the pheromone on feeding activity and to determine whether a preference for sugar or protein food sources exists (Table 2). Experiment 10.1 showed that ants consumed a significantly greater amount of both protein and protein/sugar solution than either sugar solution or control (ANOVA: $F = 27.976$, df = 3, $P < 0.001$). In Experiment 10.2, ants consumed significantly less protein solution from vials treated with 2-MeBu-diMePy in comparison to protein solution alone (*t*-test: $P = 0.008$). Experiment 10.3 showed the same effect with a sugar solution, with ants consuming significantly less sugar solution from 2-MeBu-diMePy treated feeding vials vs. sugar solution

alone (*t*-test: $P = 0.048$). Experiment 10.4 showed a non-significant decrease in the consumption of peanut butter in baits treated with 2-MeBu-diMePy compared with peanut butter alone (*t*-test: $P = 0.067$). It was noted that while pheromone-treated baits were often the first to be discovered and quickly became surrounded by ants, these ants did not readily consume the bait or appear to recruit other *W. auropunctata* at the same rate as ants at untreated baits.

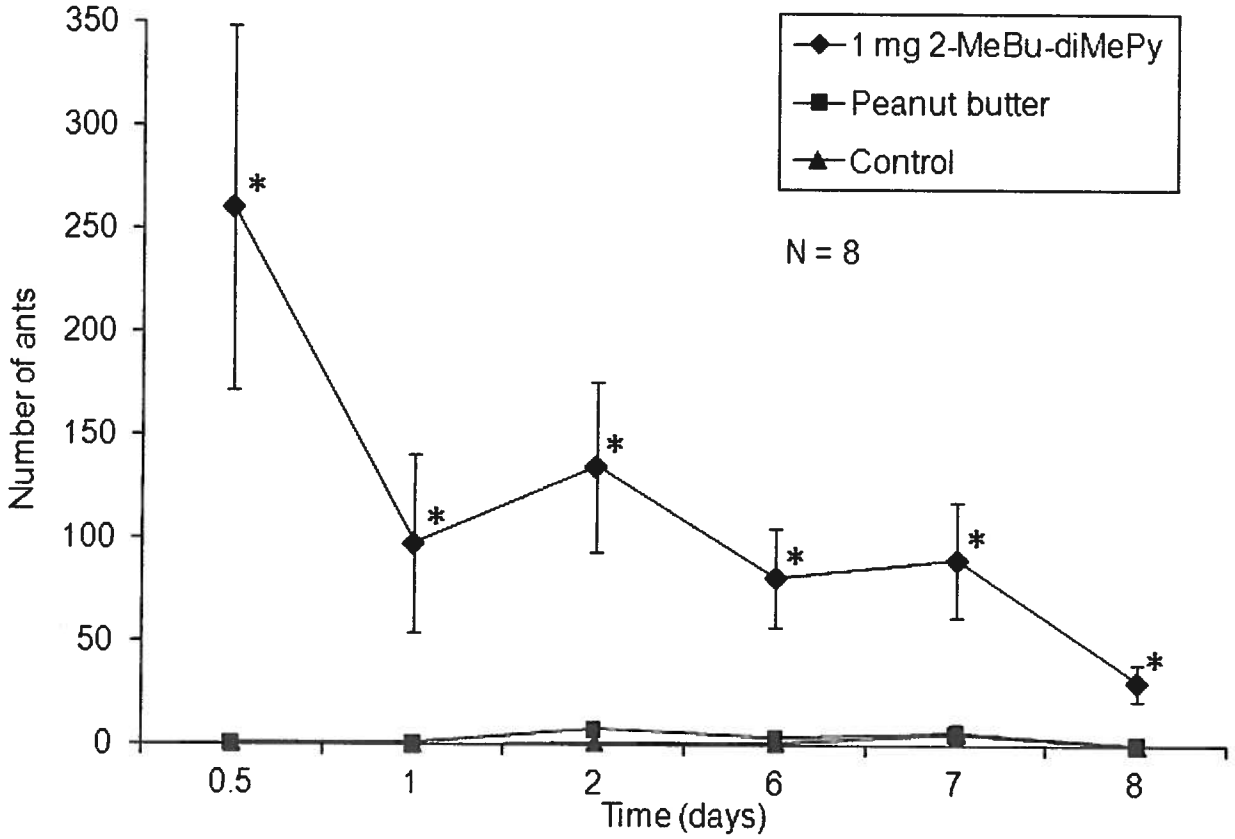

Figure 9. Experiment 4, numbers (mean ± SE) of *W. auropunctata* within 1 cm of the Tanglefoot square perimeter at given time intervals for each treatment. Asterisks identify treatments significantly different ($P < 0.05$) from others at a given time interval (ANOVA, followed by Tukey's HSD).

Table 2. Experiment 5, food material consumed by *W. auropunctata.*

	Amount consumed in grams (mean ± SE)			
Exp. 10.1*	Protein & Sugar	Protein	Sugar	Control
	1.2 ± 0.2 a	1.1 ± 0.1 a	0.14 ± 0.03 b	0.12 ± 0.02 b
Exp. 10.2†	Protein		Protein & 2-MeBu-diMePy	
	0.33 ± 0.06 a		0.14 ± 0.03 b	
Exp. 10.3†	Sugar		Sugar & 2-MeBu-diMePy	
	0.3 ± 0.1 a		0.09 ± 0.03 b	
Exp. 10.4†	Peanut Butter		Peanut Butter & 2-MeBu-diMePy	
	0.11 ± 0.05 a		0.019 ± 0.006 a	

*Means within a row followed by the same letter are not significantly different ($P > 0.05$) using Tukey's HSD test.
†Means within a row followed by the same letter are not significantly different ($P > 0.05$) using a *t*-test.

GC coinjection of synthetic 3-MeBu-diMePy with extracts of *W. auropunctata* showed the previous identification of this compound as the alarm pheromone component by Howard et al. (1982) to be incorrect. Mass spectrometry suggested the true alarm pheromone structure to be 2-MeBu-diMePy, which was confirmed by synthesis and GC analysis. Separation and identification of these two pyrazines, based on retention times and mass spectrometry has been reported in a study of cephalic compounds in the ant *Rhytidoponera metallica* (Smith) showing that separation of the compounds is possible (Tecle and others 1987).

Several factors may have contributed to the previous misidentification: the use of a relatively short, packed GC column, probably not capable of separating 3-MeBu-diMePy and 2-MeBu-diMePy; a mass spectrometry comparison performed against a limited number of alkylpyrazines. The finding by Howard et al. (1982) that the pheromone components were confined to the mandibular glands is supported by our analysis of trisected ants. Additionally,

headspace collections using SPME and Porapak Q showed that 2-MeBu-diMePy and 2-MeBu-MePy are released unaltered as active pheromone components and are not biological precursors, with 2-MeBu-diMePy being the most abundant volatile released.

In addition to being found in *W. auropunctata*, 2-MeBu-diMePy is secreted from the mandibular glands of the ants *Dinoponera australis* Emery (Oldham and others 1994), *Odontomachus bauri* Emery (Morgan and others 1999), *Rhytidoponera metallica* (Smith) (Tecle and others 1987), *Rhytidoponera victoriae* (Andre) (Brophy 1989), a *Calomyrmex* sp. (Brown and Moore 1979), and an *Ectatomma* sp. (Morgan and others 1999). Consistent with our analysis, 2-MeBu-diMePy in ants is found only in the head and has been described as an alarm pheromone in some cases. Similar alkylpyrazines, including 3-MeBu-diMePy, have been found as mandibular secretions of a phylogenetically diverse group of ants including Myrmeciinae, Myrmicinae, Formicinae and Dolichoderinae, and often as trail pheromones from Dufour's gland (Attygalle and Morgan 1984; Hölldobler and others 2001; Morgan and others 1999; Tentschert and others 2000). Despite its structural similarity to known pheromone components, 2-MeBu-MePy is novel in insects, while it has been reported as a Maillard reaction-produced component of roasted coffee volatiles (Friedel and others 1971).

While 2-MeBu-diMePy and 2-MeBu-MePy are chiral, no enantiomeric separations were achieved using a Rt-BDEXm chiral GC column. Consequently the absolute configurations of neither natural alkylpyrazine were assigned. Enantiomeric separation of a closely related pyrazine, 2-methoxy-3-(1'-methylpropyl)pyrazine, have been reported (Bungert and others 2001). The inability to separate the enantiomers of the alkyl pyrazines in the current study may be due to differences if column stationary phases or functional group differences.

Behaviors induced by alarm pheromones are often difficult to quantify (Hölldobler and Wilson 1990). While various bioassay types exist, many focus on arrestments, attraction, increased mobility, and repulsion. To test the alarm pheromone behaviors of *W. auropunctata* elicited by racemic 2-MeBu-diMePy and 2-MeBu-MePy, we chose a circle bioassay modified from Howard et al. (1982), Vander Meer et al. (1988), and Fujiwara-Tsujii et al. (2006). This assay allowed quantification of arrestment, attraction, and increased mobility behaviors.

Higher numbers of crossings in the bioassays indicate a combination of increased ant mobility and attraction, although the assays could not distinguish between these two characteristics of alarm behavior. Bioassay results generally showed that the numbers of crossings induced by synthetic pyrazines were significantly greater than those induced by the negative control, while they were not significantly different from the positive control. These behaviors are consistent with the assignment of 2-MeBu-diMePy and 2-MeBu-MePy as alarm pheromone components.

Compared to crossings, arrestment data showed greater variation both among treatments and between bioassay types. Importantly, crushed ant treatments produced significantly higher arrestment numbers than did synthetic pyrazine treatments in all bioassays except Experiment 1. This observation could be due to as yet unidentified pheromone compounds in the ant and/or the method of pyrazine presentation in the bioassays. Howard et al. (1982) hypothesized that alkylpyrazines attract *W. auropunctata*, while less volatile compounds contribute to arrestment. Headspace analysis by SPME and Porapak Q found that 2-MeBu-diMePy is the major volatile component released by *W. auropunctata* workers, with 2-MeBu-MePy and other volatiles present in small amounts. In addition, observed trail following behavior and the presence of additional volatile compounds in whole-ant extracts could indicate the presence of other pheromones that

might contribute to ant arrestment. In support of this idea, extracts of separated ant heads showed 2-MeBu-diMePy to be the major volatile component while other volatile compounds were found to be confided to the gaster extracts.

However, bioassay experiments suggested that pheromones from parts of the ant other than the head do not contribute to increased arrestment. Experiments 3, 4, and 5 confirmed that increased arrestment is due to a characteristic of the ant head, and not other body segments or their components: positive controls for Experiments 3 and 4 (whole ant and crushed head respectively) produced similar results and in Experiment 5, the crushed head arrested significantly more ants than did the crushed thorax or crushed gaster. If yet unidentified compounds account for increased arrestment by ant heads, they are likely nonvolatile (i.e. cuticular hydrocarbons) or present in trace amounts.

Another possible explanation for high arrestment by crushed ant may be the presentation of pheromone components in an optimum concentration and/or synergistic formulation. Experiment 2 tested a dilution series of the biologically relevant 100:1 formulation of 2-MeBu-diMePy/2-MeBu-MePy but found no synergy or dose response at the concentrations and formulation tested.

Variations in behavioral responses may also be the result of bioassay differences between the smaller arena (Experiments 1 and 2) and the larger arena (Experiments 3, 4, and 5). Interestingly, the most active pyrazine appears to be bioassay dependent, with 2-MeBy-MePy most active in the smaller arena, while 2-MeBy-diMePy is most active in the larger arena. These differences may arise from a number of different factors. The smaller arena, used in Experiments 1 and 2, may be biased toward shorter-range attraction, as ants were removed from their colony areas and placed in a small container. In contrast, Experiments 3, 4, and 5 were conducted in the

foraging area of the colony with little to no disturbance due to moving ants between containers. Furthermore, Experiments 3 and 4 were conducted with many more ants, perhaps exaggerating the differences in arrestments compared to Experiment 1.

Additionally, without assignment of the absolute configuration of the natural *W. auropunctata* alkyl pyrazines, strict comparisons between the natural pheromone and the racemic synthetic pyrazines are complicated. Absolute configurations of pheromones can cause profound differences in bioactivity as illustrated with the leaf-cutting ant, *Atta texana* (Riley and others 1974). The principal alarm pheromone of this ant is (*S*)-4-methyl-3-heptanone, which was shown to be ~100 times more active than its corresponding (*R*)-isomer, demonstrating for the first time the dependence of pheromone bioactivity on absolute configuration. Additionally, the (*R*)-isomer did not inhibit responses to the (*S*)-isomer at the ratios tested. Illustration of the complex relationship between pheromone chirality and bioactivity are demonstrated by examples such as inhibition by the wrong enantiomers, blends of enantiomers (includeing racemic pheromones) and differential responses between males and females (Mori 2007). Establishing what effect, if any, chirality has in the alarm signaling of *W. auropunctata* will require assigning the absolute configurations of both ant alkyl pyrazines, and further bioassays with the enantiomers of 2-MeBy-diMePy and 2-MeBy-MePy.

Studies have shown a preference exhibited by *W. auropunctata* for peanut butter over other food sources, including honey, pineapple juice, and tuna oil (Williams and Whelan 1992). Consequently, peanut butter is a commonly used bait for detection of *W. auropunctata* (Causton and others 2005; Kirschenbaum and Grace 2007). It is interesting to note that along with containing sugars, lipids, and proteins which make it a desirable food source, peanut butter also releases a number of volatile alkylpyrazines, including 2,5-dimethylpyrazine and 2,5-dimethyl-3-

ethylpyrazine (Joo and Ho 1997). The structural similarity of these pyrazines to the little fire ant alarm pheromone, along with the feeding preference shown for peanut butter, warrants an investigation into the attractiveness of peanut butter volatiles to *W. auropunctata.*

2-MeBu-diMePy and 2-MeBu-MePy attracted and arrested more ants than controls in every field test performed. While the relative importance of attraction and arrestment behavioral modalities is difficult to quantify in Experiments 6-9, both are likely to have contributed to the number of ants counted in defined areas. Attraction appeared to be most important in Experiments 6-8, while many of the ants counted in Experiment 4 seemed to be arrested after an initial attraction.

2-MeBu-diMePy is the primary alarm pheromone component found in *W. auropunctata* and therefore was the initial focus of this field research. Increasing amounts of 2-MeBu-diMePy in Experiment 6 showed an attraction and arrestment dose response by *W. auropunctata* workers with a maximum response to the 1 mg lure (Fig. 1). This amount of pheromone is considerably higher than the ~200 ng of the alarm pyrazine that Howard *et al.* (1982) found in individual *W. auropunctata* workers. However, we did not quantify the absolute amount of 2-MeBu-diMePy released per ant when alarmed or the release rates of the lures in our study. Therefore, strict comparisons between the activity of 2-MeBu-diMePy and the alarm pheromone released by ants are not possible. A reasonable assumption may be that at least the 1 mg dose releases amounts of pheromone some orders of magnitude above that which is likely to be encountered by ants naturally. Irrespective of the relative activity of various concentration of 2-MeBu-diMePy, the attractiveness of this compound makes it a good candidate for use in monitoring *W. auropunctata* populations.

Both alarm pheromone components previously identified in *W. auropunctata* (Showalter and others 2009a) were shown to be behaviorally active in laboratory bioassays. However, the bioassay results did not consistently indicate either a most attractive pyrazine or blend of pyrazines. Such pheromone attraction is often mediated by multiple chemical components, and the ability to produce a blend of chemical signals allows for greater specificity in intra-species communication, complex chemical messages, and the possibility of enhanced signaling through synergistic effects (Hölldobler and Wilson 1990). Although signal complexity is common in nature, studying the interactions of component compounds can be difficult, particularly when concentration and component ratios may need to be adjusted to find an optimal attractant blend. An example of this complexity can be seen in the trail pheromone of the ant *Tetramorium meridionale* Emery, whose trail pheromone's four components are required in combination to reproduce activity similar to live ant secretions (Jackson and others 1990).

To test the synergistic effects of 2-MeBu-diMePy and 2-MeBu-MePy (Experiment 7), we examined their relative attractancy by testing these pyrazines singly and in blends based on ratios found in *W. auropunctata* workers (Showalter and others 2009b). There was no significant difference in attraction between pheromone components at any of their tested ratios, pointing to a lack of synergistic effects between the components. This is unusual since examples of pheromone synergism predominate in the literature; however, non-synergistic interactions have also been reported. An example is the gregarious desert locust *Schistocerca gregaria* (Forskål), which produces an oviposition aggregation pheromone consisting of two major components that are equally active individually and in combination, and thus not synergistic (Rai and others 1997).

Experiment 8 was designed to compare the attractiveness of 2-MeBu-diMePy to peanut butter, a bait commonly used to survey for *W. auropunctata*. Peanut butter releases a number of volatile alkylpyrazines, notably 2,5-dimethylpyrazine and 2,5-dimethyl-3-ethylpyrazine (Joo and Ho 1997), which are similar in structure to the pyrazines found in *W. auropunctata*. Surprisingly, given the release of alkylpyrazines and previous reports of attractancy (Williams and Whelan 1992), peanut butter was not found to attract significantly higher numbers of ants than the negative control. This was further supported by the results of Experiment 9, which again showed peanut butter did not attract more ants than the negative control. This discrepancy with previous reports (Williams and Whelan 1992) probably results from differences in bioassay methodology. Observations of peanut butter discovery by ants, during Experiment 10.4, indicate that *W. auropunctata* workers quickly swarm the food resources once they are discovered, but that initial discovery is achieved through somewhat random searching and is not aided by volatile cues. This conclusion is most strongly supported by the results of Experiment 9, in which ants were prevented from contacting the peanut butter by the surrounding Tanglefoot, and in which case no attraction was observed. The strong preference shown for peanut butter (Williams and Whelan 1992) is likely due to recruitment mediated by the ants themselves (e.g. physical and pheromonal recruitment).

The original purpose of Experiment 9 was to assess whether 2-MeBu-diMePy could be used in combination with Tanglefoot to monitor *W. auropunctata* populations without constant observation. However, *W. auropunctata* were cautious when approaching lures surrounded by Tanglefoot and did not become entangled. This result presents serious difficulties in using 2-MeBu-diMePy as a detection tool. However, continuing observations of these lures showed that they attracted and arrested ants for up to 8 days (Fig. 3). This longevity compares favorably with

lures currently used for detection, such as peanut butter, which often deteriorate when in the field for more than a few days.

Of primary interest in researching *W. auropunctata* pheromones is their potential use in monitoring and controling this invasive pest. One possible control application would be the inclusion of 2-MeBu-diMePy in insecticidal baits to increase feeding (Hughes and others 2002). In Experiments 10.2 and 10.3, however, ants consumed significantly less sugar and protein from feeding vials treated with 2-MeBu-diMePy than from those with protein or sugar alone. These results were not consistent with previous studies of ant pheromones, which increased or did not affect consumption of baits treated with pheromone (Greenberg and Klotz 2000; Hughes and Goulson 2002; Hughes and others 2002; Robinson and Cherrett 1978).

Alarm and trail pheromones as bait enhancer candidates have consistently been shown to induce attraction (Greenberg and Klotz 2000; Hughes and others 2002). However, consumption of bait does not always increase proportionally with the number of ants attracted, as has been shown through ant responses to alarm pheromones. Bait removal is likely to increase merely because a larger number of ants in an area increases the likelihood that more bait will be removed, but the increase is not always proportional to the number of ants attracted (Robinson and Cherrett 1978). In our results, however, the pyrazine actually decreases bait removal, suggesting that including the alarm pheromone in *W. auropunctata* baits is not necessarily an effective way to increase bait consumption and therefore aid in ant control.

The decrease in the amount of bait consumed by ants at pheromone-treated stations suggests that the alarm pheromones may inhibit normal responses to a food resource. This is particularly supported by the observation that *W. auropunctata* workers seemed to find treated baits more quickly, but do not recruit other ants to the same extent as workers at untreated baits.

A hierarchy of behavior may exist by which some behaviors supersede others once elicited. In this case, alarm behavior may take precedence over feeding or retrieval of food items. An example of this behavioral hierarchy was noted by Moser *et al.* (1968) and Blum *et al.* (1968) who both observed that *Atta* spp. often dropped their loads or were less likely to transport bait when alarmed by synthetic pheromone. Hughes and Goulson (2001) present another possible reason that alarm pheromone-treated baits can be more attractive but less consumed. In a study with grass-cutting ants, they found that the main caste to respond to an alarm pheromone is the minor worker, which may be too small to transport bait (Hughes and Goulson 2001). Size is unlikely to be a problem for *W. auropunctata*, however, because it appears to have only one worker caste, which is capable of carrying the bait used in our studies.

Although our field tests showed the *W. auropunctata* alarm pheromone to be not particularly useful for increasing consumption of food baits or trapping ants with Tanglefoot, they revealed that 2-MeBu-diMePy is significantly more attractive than peanut butter. This quality may be instrumental in developing other control applications, such as direct disruption of trail following behavior (Suckling and others 2008) or a different trapping device for detection.

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Dissemination of Results:

Publications

Showalter, D. N., E. J. Troyer, M. Aklu, E. B. Jang, M. S. Siderhurst. 2010. Alkylpyrazines: Alarm pheromone components of the little fire ant, *Wasmannia auropunctata* (Roger) (Hymenoptera: Formicidae). Insectes Sociaux – in press.

Troyer, E. J., N. T. Derstine, D. N. Showalter, E. B. Jang, M. S. Siderhurst. 2009. Field studies of *Wasmannia auropunctata* (Roger) alkylpyrazines: Towards management applications. Sociobiology 54(3) 955-971.

Oral Presentations

Alkylpyrazines: Alarm pheromone components of the little fire ant, *Wasmannia auropunctata* (Roger) (Hymenoptera: Formicidae). Elisa J. Troyer, David N. Showalter, Eric B. Jang, Matthew S. Siderhurst. 2010 Entomological Society of America, Eastern Branch Meeting, Annapolis, MD.

Field studies of *Wasmannia auropunctata* (Roger) alkylpyrazines: Towards management applications. Nathan T. Derstine, Elisa J. Troyer, David N. Showalter, Eric B. Jang, Matthew S. Siderhurst. 2010 Entomological Society of America, Eastern Branch Meeting, Annapolis, MD.

Loading pheromone lures in the field


Counting area on macnut tree

LFA responding to a 1 mg 2,5-dimethyl-3-(2-methylbutyl)pyrazine lure

Protein solution feeding station

LFA feeding at a protein solution station

LFA worker