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Differential Field Responses of the Little Fire Ant, *Wasmannia auropunctata* (Roger), to Alarm Pheromone Enantiomers

Yang Yu · Eric B. Jang · Matthew S. Siderhurst

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Abstract The little fire ant, *Wasmannia auropunctata* (Roger) (Hymenoptera: Formicidae), is an invasive ant with negative impacts on both biodiversity and agriculture throughout the tropics and subtropics. Field experiments were conducted in order to elucidate the relative attractiveness of the enantiomers of the alarm pheromones, 2,5-dimethyl-3-(2-methylbutyl)pyrazine and 3-methyl-2-(2-methylbutyl)pyrazine. The enantiomers tested were synthesized from commercially available (*S*)-2-methylbutan-1-ol or kinetically resolved (*R*)-2-methylbutan-1-ol, prepared using *Pseudomonas cepacia* lipase (PCL). Bioassays conducted in a macadamia orchard on the island of Hawaii demonstrated that *W. auropunctata* were preferentially attracted to the (*S*)-enantiomers of both alkyl pyrazines over the racemic mixtures in all experiments. To our knowledge, this is the first instance of differential attraction of ants to the enantiomers of chiral pyrazine pheromones despite many examples of these compounds in the literature. In addition, using a chiral column it was determined that (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine and (*S*)-3-methyl-2-(2-methylbutyl)pyrazine are the only enantiomers produced by *W. auropunctata*.

Keywords Alkylpyrazines · *Pseudomonas cepacia* lipase · Chiral column · Passive behavior response · Hymenoptera · Formicidae · Invasive pest · Chirality · Pyrazines

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Introduction

Considered one of the worst invasive pest ants (Lowe et al. 2000), *Wasmannia auropunctata* (Roger) (Hymenoptera: Formicidae) negatively impacts both biodiversity and agriculture (Le Breton et al. 2003; Walker 2006; Wetterer and Porter 2003). While the distribution of this ant is mostly pantropical, greenhouse infestations have been reported as far north as Canada and the United Kingdom (Jourdan et al. 2002; Wetterer and Porter 2003). The impact of *W. auropunctata* seems to vary somewhat with location. For example, despite having been present in Florida for over 75 years, *W. auropunctata* is not considered a major pest there. However, on invaded Pacific islands, this ant has had a much larger ecological and economic impact (Wetterer and Porter 2003).

While *W. auropunctata* workers are rather slow-moving and diminutive in size, the potent venom from their stings is painful, and they have become an important deterrent to farm laborers that harvest infested crops (Conant 2000; Smith 1965). They also affect agriculture by tending homopterans, which directly damage crops and may vector disease (de Souza et al. 1998; Spencer 1941). Ecological impacts include the stressing of vertebrates (Wetterer 1997; Wetterer et al. 1999), reduction of invertebrate populations (Ulloa-Chacón et al. 1991), and displacement of native ants (Le Breton et al. 2003; Walker 2006).

Trapping is an important aspect of the pest management of *W. auropunctata*, particularly in detecting and monitoring this pest. Trapping methods often are improved with semiochemical attractants, such as pheromones, which in many cases attract a limited number of species. In the case of *W. auropunctata*, two components of the alarm pheromone produced by the ant have been identified as 2,5-dimethyl-3-(2-methylbutyl)pyrazine (2-MeBu-diMePy) and 3-methyl-2-(2-methylbutyl)pyrazine (2-MeBu-MePy) (Showalter et al. 2010). In field and laboratory bioassays, both pyrazines

induced attraction and arrestment, and increased locomotion (Showalter et al. 2010; Troyer et al. 2009). Additionally, field trapping demonstrated the usefulness of traps for monitoring *W. auropunctata* (Derstine et al. 2012).

Chirality can play an important role in insect pheromone communication, although determination of stereochemistry often is a challenge (Mori 2007). Ant alarm pheromones hold an important place in the study of insect pheromone stereoisomers because the alarm pheromone of the leaf-cutting ant *Atta texana*, (*S*)-4-methyl-3-heptanone, was the first insect semiochemical for which a differential response to the enantiomers was demonstrated (Riley et al. 1974). 4-Methyl-3-heptanone with an enantiomer ratio of 4:1 (S/R) also elicited trail-following activity in *Aphenogaster albisetosus* (Holldobler et al. 1995), while in *A. cockerelli* only the (*S*)-enantiomer was detected in venom glands. For the latter species, this compound did not induce trail-following behavior but caused the workers to leave the nest. Chirality also has been observed in components of the venom gland of *Leptogenys peuqueti* (Janssen et al. 1997) from which a group of alcohols and their acetates, with two or three stereocenters, has been identified. However, only the (4*R*,10*R*) and the (4*S*,10*S*) chiral forms of 4,10-dimethyl-7-tridecanol and their acetates have been tested, and the stereochemical composition of the pheromone components is yet to be established. A further example of the importance of chirality is seen in *Camponotus* spp. with Bestmann et al. (1999) having shown that (2*S*,4*R*,5*S*)-2,4-dimethyl-5-hexanolide is the active trail pheromone out of four pairs of possible enantiomers.

One unresolved aspect of the *W. auropunctata* alarm pheromone is what role pyrazine chirality plays in this communication system. In our earlier work, it proved difficult to determine the enantiomeric ratios of the natural alkylpyrazines 2-MeBu-diMePy and 2-MeBu-MePy using GC analysis, even though different chiral GC columns, multiple temperature profiles, and sampling and injection techniques were tried (Showalter et al. 2010). Given this difficulty in directly identifying the absolute configurations of the alarm pheromones, another approach to provide insight into the *W. auropunctata* alarm pheromone communication system, would be the synthesis and comparison of behavioral responses to all pyrazine enantiomers. Key to the preparation of these enantiomers is the synthesis of the chiral 2-methylbutyl side chain moiety. While intermediates having the (*S*)-moiety currently are commercially available in the form of either the alcohol or the bromide, the (*R*) counterparts are not, although several synthetic routes have been reported previously. Sasaerila et al. (2000) reported a resolution of diastereoisomers derived from manipulation of the racemic alcohol on a chiral column. Alternatively, a one-step synthesis by reducing the corresponding enantiomerically pure acid was reported by Mori (2009), but this alkyl acid currently is neither commercially available nor readily easy to synthesize. For the current study, lipases were chosen for the synthesis as they have been shown to be versatile tools in the resolution of primary alcohols alpha or beta to a stereocenter (Ghanem and Aboul-Enein 2005; Heinsman et al. 1998, 2002).

In this paper, we report the synthesis and field evaluation of both enantiomers of 2-MeBu-diMePy and 2-MeBu-MePy, synthesized from either commercially available (*S*)-2-methylbutan-1-ol, or (*R*)-2-methylbutan-1-ol. The latter was prepared by kinetic resolution of the racemate using a *Pseudomonas cepacia* lipase (PCL). Field assays demonstrated that *W. auropunctata* were preferentially attracted to the (*S*)-enantiomer over both the racemic mixture and the (*R*)-enantiomer. Additionally, we were also able to resolve the natural pheromones using a γDEX120 chiral column. This analysis showed that (*S*)-2-MeBu-diMePy and (*S*)-2-MeBu-MePy were the only enantiomers produced by *W. auropunctata* within the limits of detection. To our knowledge, this is the first instance of differential attraction of ants to the enantiomers of chiral pyrazine pheromones.

Methods and Materials

Instrumentation Synthetic pyrazines and their intermediates were analyzed by gas chromatography (GC) using an Agilent Technologies 6890 N gas chromatograph interfaced to a Hewlett Packard 5973 Mass Selective Detector equipped with an HP-5MS column (30 m×0.25 mm ID, 0.25 μm film thickness). The standard temperature program used was 80–220 °C at 10 °C/min with a 1 min start delay. The injector temperature was set at 250 °C, and helium was used as carrier gas (1.1 ml/min).

The enantiomeric excess (ee) of 2-methylbutan-1-ol (**3**) was determined by GC with a Rt-βDEXm chiral column (30 m×0.25 mm ID, 1.0 μm film thickness; Restek Corporation, Bellefonte, PA, USA) installed into an Agilent Technologies 6890 N gas chromatograph. The optimal temperature program used was 40 °C held for 15 min then increased to 100 °C at a rate of 20 °C/min.

The enantiomeric excess of 2-MeBu-diMePy enantiomers (**1**) and 2-MeBu-MePy (**2**) were determined using a γDEX120 chiral column (30 m×0.25 mm ID, 1.0 μm film thickness; Supelco, Bellefonte, PA, USA) installed into an Agilent Technologies 6890 N gas chromatograph. The optimal temperature program used for the resolution of **1** was 85 °C held for 80 min then increased to 150 °C at a rate of 10 °C/min. The temperature program for **2** was 55 °C held for 130 min then increased to 220 °C at a rate of 10 °C/min. The injector temperature was set at 250 °C, and helium was used as carrier gas (4.0 ml/min).

^{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. Optical rotation was measured using an Autopol®IV Automatic Polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA).

Synthesis All reactions were carried out under an argon atmosphere. Syringes and cannulas were used to transfer reagents and solvents. All solvents and reagent compounds were purchased from Sigma–Aldrich, Inc., Saint Louis, MO, USA, or Fisher Scientific, Rochester, NY, USA, and were used without further purification, with the exception of diethyl ether, which was dried by distillation from sodium/benzophenone. Thin layer chromatograms of compounds were visualized under UV and with iodine.

*Kinetic Resolution of (±)-2-Methylbutan-1-ol (**3**)* The method of Wang et al. (2009) was used with modifications to resolve the alcohol using *Pseudomonas cepacia* lipase (PCL). Lipase (10 g) was added to a solution of (±)-2-methylbutan-1-ol (40 g, 0.45 mol) in ether (250 ml), and the mixture was stirred for several minutes before the addition of succinic anhydride (43.6 g, 435 mmol), followed by stirring overnight. Solids formed during the reaction were filtered, and the organic phase was washed with Na_2CO_3 (1 M), water and brine. The filtrate was dried with $MgSO_4$, filtered, and concentrated to give an organic layer (8 g). (*R*)-2-Methylbutan-1-ol was purified by vacuum distillation (82–85 °C, 8 mmHg) yielding 1.7 g of a clear colorless liquid (98 % ee based on GC-FID analysis; Wang et al. (2009) reported 98 % ee).

*(R)-1-Bromo-2-Methylbutane ((R)-**4**)* Neat PBr_3 (19.0 g, 70.3 mmol) was added dropwise at −10 °C to (*R*)-**3** (6.79 g, 77.2 mmol). The reaction solution was warmed slowly to 0 °C over 1 h and then to room temperature, at which temperature it was stirred for 2 h. The reaction mixture was then heated at 100 °C for 20 min before being cooled to 0 °C and quenched with ice. The reaction mixture was diluted with ether and then washed with water and $NaHCO_3$ until a pH of 9 was reached or no bubbles were emitted when $NaHCO_3$ was added. The organic layer was washed with brine, dried with $MgSO_4$, and filtered through a sintered funnel. The filtrate was concentrated to give a clear colorless liquid (11.4 g, 98 % yield). (*S*)-**4** was prepared from commercially available (*S*)-2-methyl-1-butanol in a similar manner to (*R*)-**4** as described above. (rac)-**4** was obtained from a commercial source.

*(R)-3,5-Dimethyl-3-(2-Methylbutyl)pyrazine ((R)-**1**)* Magnesium turnings (0.84 g, 35 mmol) were placed in a three-neck round-bottom flask with a condenser, and the system was flame dried under argon purge. After it was cooled to room temperature, diethyl ether (4 ml) was added followed by a portion of bromide (**4**, 0.7 ml) at once. The remaining bromide (**4**, 2.5 ml, in 29.2 ml ether, total 29.2 mmol) was added dropwise to maintain a gentle reflux. An ice bath was used to cool the reaction vessel when refluxing became too vigorous. The reaction mixture was heated at 70 °C for 30 min after the addition and then cooled to room temperature. The product was used in the next step without further purification.

An approximate amount of dichloro[1,3-bis(diphenylphosphino)propane]nickel(II) (DPPP-$NiCl_2$) was transferred into a flame-dried, round-bottom flask without weighing, and back calculated to be 222 mg (0.41 mmol). 3-Chloro-2,5-dimethylpyrazine (1.2 ml, 10 mmol) was added at 0 °C, followed by diethyl ether (5 ml). The suspension was stirred for 10 min before the Grignard reagent from (*R*)-**4** was added dropwise. After addition, the reaction mixture became deep red to black, and was stirred for 30 min before it was warmed to room temperature. A yellow powdery solid started to precipitate and made it difficult to stir. The reaction was cooled to 0 °C and quenched by addition of diethyl ether and ice after stirring for 4 h at room temperature. The reaction mixture was washed with water, brine, and HCl (1 M). The aqueous layer was extracted with diethyl ether. The combined organic layers were dried with $MgSO_4$ and concentrated. The residue was purified by silica gel column chromatography, (Et_2O / pentane – 1:4) to give (*R*)-**1** (1.53 g, 93 % yield, = −4.97° (c =1.41, $CHCl_3$)). The ^{1}H-NMR and ^{13}C-NMR were as previously reported by Showalter et al. (2010). (*S*)-**1** (= + 6.62° (c =3.02, $CHCl_3$)), (*R*)-**2** (= −8.99° (c =1.49, $CHCl_3$)), and (*S*)-**2** (= +10.4° (c =1.27, $CHCl_3$)) were prepared in a similar manner as described above.

Insects and Field Location All field tests were conducted in a macadamia nut orchard on the island of Hawaii, outside Papaikou, HI (GPS coordinates: 19.787029, −155.124443), from 9:00 to 13:00 in June 2010 and November 2013. Tests were performed in macadamia nut trees where *W. auropunctata* trails were readily observed. Hourly air temperatures and relative humidity data were obtained from a Natural Resources Conservation Service database, USDA (scan site: Island Dairy, Papaikou).

Ants used for pheromone analysis were collected from macadamia trees using a portable vacuum cleaner equipped with a capture chamber fashioned from an extra coarse fritted funnel. The thin neck of the funnel was attached to the vacuum with tubing, while a rubber stopper was inserted into the larger funnel opening ("top") fitted with a glass tube (5 mm diam) which was used for collecting ants. Approximately 20 ml ants was collected and soaked with pentane (40 ml) overnight. The ants were removed by filtration, and the pentane filtrate was concentrated to give a brown liquid (100 mg). The extract was applied to silica gel (5 ml) in a pipette and eluted with pentane (10 ml), 5 % Et_2O/pentane (15 ml) and 20 % Et_2O/pentane (15 ml), and fractions of 5 ml were collected. Fraction 8 contained compound **1** according to GC/MS analysis and analyzed with GC-FID using the γDEX120 chiral column. Because of the low concentration of minor component 2-

MeBu-MePy (**2**), GC/MS analysis was performed on ant samples collected previously and extracted in CH_2Cl_2 without fractionation.

Bioassays Field bioassays were conducted as previously described by Troyer et al. (2009). Four map pins were used to define a 4×4 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, USA) was pinned to the center of each counting area. Counting areas were limited to one per tree and remained fixed throughout all experiments. Chemicals were dissolved in CH_2Cl_2 at 20 mg/ml and 50 μl (1 mg loading) were applied to a rubber septum. Controls were treated with CH_2Cl_2 (50 μl). Rubber septa with treatments were placed on the center of the square after the solvent had evaporated. Treatments were randomized using Latin square designs, so that each tree/ant trail was exposed to every treatment. An interval of at least 10 min was allowed between successive treatments during which time ants vacated the test areas and returned to the nearby trails.

Experiment 1 examined the relative attractiveness of the (*S*)-enantiomers and the racemic mixtures of the two pyrazines. These assays were conducted on 17 June 2010 before the (*R*)-pyrazine enantiomer had been prepared. Ants within the marked areas were counted at 5-min intervals for 30 min. Hourly temperatures varied from 23 to 26 °C and relative humidity from 55 to 68 %.

Experiment 2 examined the relative attractiveness of both enantiomers and the racemate of the *W. auropunctata* dimethylpyrazine pheromones of 2-MeBu-diMePy. These assays were conducted on 14 November 2013. Ants within the marked areas were counted at 4-min. intervals for 28 min. Hourly temperatures varied from 22 to 24 °C, and relative humidity was from 81 to 82 %.

Experiment 3 examined the relative attractiveness of both enantiomers and the racemate of the *W. auropunctata* monomethylpyrazine pheromones of 2-MeBu-MePy. These assays were conducted on 21 November 2013. Ants within the marked areas were counted at 4-min. intervals for 28 min. Hourly temperatures varied from 22 to 25 °C, and relative humidity was from 66 to 78 %.

Statistical Analyses Each ant count was transformed to $\sqrt{(x+0.1)}$ to normalize all data sets, and these were analyzed using ANOVA followed by Tukey's HSD test (alpha=0.05) to compare means using JMP 10.0.0 software (SAS Institute Inc. Cary NC, USA). All analyses of significance were made at the $P<0.05$ level. Data from Experiment 1 were analyzed first with all treatments included and secondarily by pyrazine, comparing among monomethylpyrazines and the control, and comparing among the dimethylpyrazines and the control.

Results

Synthesis Enantiomers of 2-MeBu-diMePy (**1**) and 2-MeBu-MePy (**2**) were synthesized from the corresponding enantiomers of 2-methylbutan-1-ol (**3**) by bromination with PBr_3 and conversion to Grignard reagents, followed by coupling with the appropriate chloropyrazines catalyzed by DPPP-$NiCl_2$ (Fig. 1). Two lipases, porcine pancreatic lipase and *Pseudomonas cepacia* lipase (PCL), were evaluated for kinetic resolution of (**3**) according to previous reports by Wang et al. (2009) and Yu and Plettner (2013). The highest enantiomeric excess (ee) obtained with porcine pancreatic lipase was less than 30 %. However, PCL gave the (*R*)-alcohol in 98 % ee as determined by GC-FID on the Rt-βDEXm column. The E value of the PCL was 8, giving a yield of only 2 %, but this procedure provided a one-pot reaction from readily available starting materials to obtain (*R*)-(**3**) in high ee (98 %).

Stereochemical Analysis Although the enantiomers of pyrazines (**1**) and (**2**) could not be separated by Rt-βDEXm or Lipodex-E chiral GC-columns in previous work (Showalter et al. 2010), the chiral intermediate, 2-methyl-1-butanol (**3**), gave near base line separation on an Rt-βDEXm column. However, using a γDEX120 chiral column, base-line resolution of enantiomers of dimethylpyrazine (**1**) [(*R*)-2-MeBu-diMePy 61.40 min, (*S*)-2-MeBu-diMePy 62.51 min] and a moderate resolution of enantiomers of methylpyrazine (**2**) [(*R*)-2-MeBu-MePy 113.4 min, (*S*)-2-MeBu-MePy 116.3 min] were achieved (Fig. 2). The synthetic (*R*)-2-MeBu-diMePy was determined to be 84 % ee by analysis on the γDEX120 chiral column.

Analyses of the pyrazines from *W. auropunctata* ants on the γDEX120 column showed that both the 2-MeBu-diMePy and 2-MeBu-MePy had the (*S*)-configuration (Fig. 2).

Field Bioassay of Pyrazine Enantiomers The relative attractiveness of the *W. auropunctata* mandibular (*S*)-enantiomer and racemic pyrazines were measured in Experiment 1 (Fig. 3). When considering all treatments together, the number of ants observed in counting areas was greatest with the (*S*)-2-MeBu-diMePy and (*S*)-2-MeBu-MePy treatments, followed by the racemic treatments and then the control. However, there were no clear significant differences between treatments, so a second analysis was conducted comparing each set of alkyl substituted pyrazines and the control (Fig. 3, 2-MeBu-diMePy indicated with lower letters, 2-MeBu-MePy indicated with Greek letters). For both pyrazines, the (*S*)-enantiomer was most active with the racemic being more active than the control (comparison between all: ANOVA: $F=33.5$; $df=4,595$; $P<0.001$; between isomers of 2-MeBu-diMePy: ANOVA: $F=60.8$; $df=2,357$; $P<0.001$; between isomers of 2-MeBu-MePy: ANOVA: $F=40.0$; $df=2,357$; $P<0.001$).

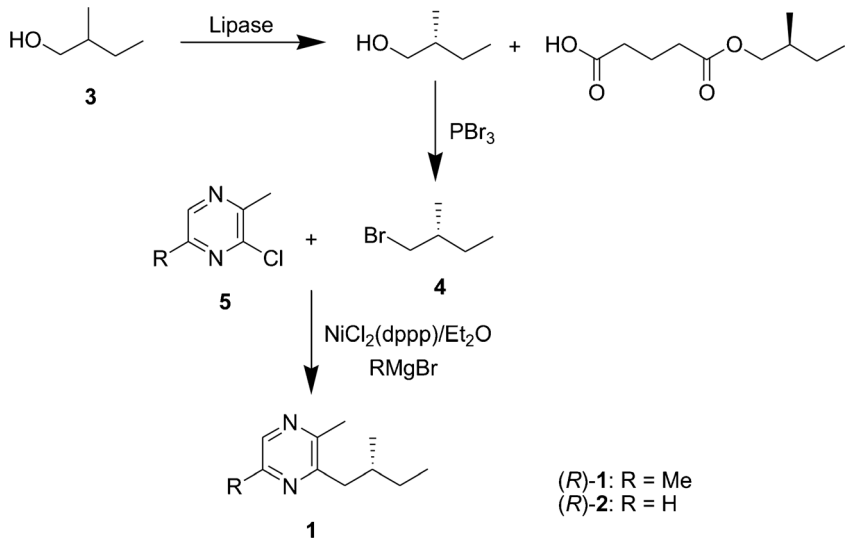

Fig. 1 Synthesis of (*R*)-2,5-dimethyl-3-(2-methylbutyl) pyrazine (**1**) and (*R*)-3-methyl-2-(2-methylbutyl)pyrazine (**2**)

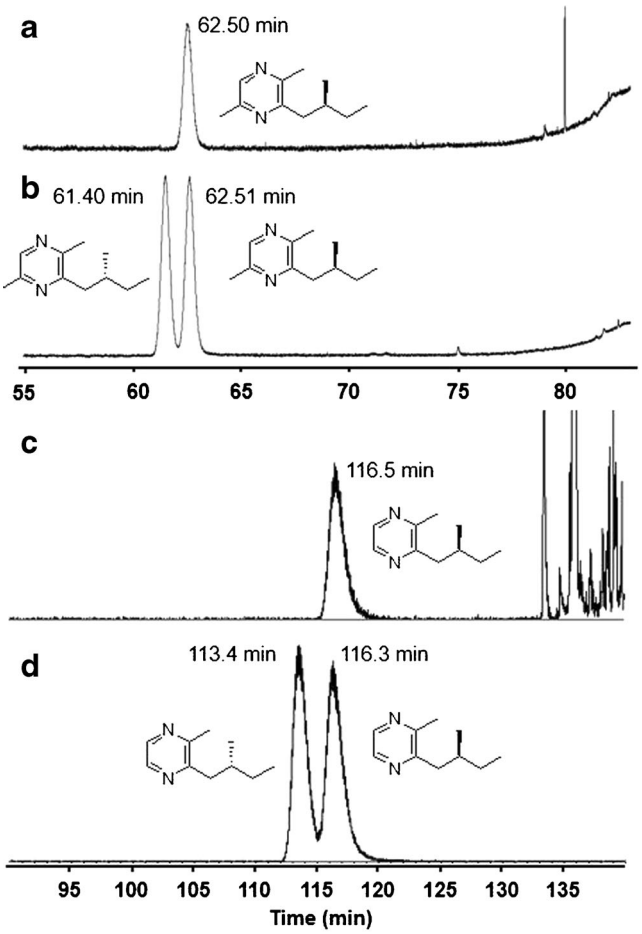

Fig. 2 Enanatioselective gas chromatograms of (a) naturally occurring (*S*)-3,5-dimethyl-3-(2-methylbutyl)pyrazine from *Wasmannia auropunctata* workers ((*S*)-**1**), (b) synthetic racemic 3,5-dimethyl-3-(2-methylbutyl)pyrazine ((±)-**1**), (c) naturally occurring (*S*)-3-methyl-2-(2-methylbutyl)pyrazine from *W. auropunctata* workers ((*S*)-**2**), and synthetic racemic 3-methyl-2-(2-methylbutyl)pyrazine ((±)-**2**) (γDEX120 column)

Experiments 2 and 3 measured the relative attractiveness of each enantiomer and the racemic *W. auropunctata* mandibular pyrazines (Figs. 4 and 5). As in Experiment 1, the (*S*)-enantiomers again attracted significantly higher numbers of ants, followed by the racemate, (*R*)-enantiomer, and unbaited control (Experiment 2 (2-MeBu-diMePy): ANOVA: $F=54.0$; $df=3,528$; $P<0.001$, Experiment 3 (2-MeBu-MePy): ANOVA: $F=36$; $df=3,528$; $P<0.001$). (*S*)-2-MeBu-diMePy attracted 1.5-fold more ants than the racemate, while the racemate attracted 1.9-fold more than (*R*)-2-MeBu-diMePy. (*S*)-2-MeBu-MePy attracted 2.5-fold more ants than its racemate, which in turn attracted 2.4-fold more than (*R*)-2-MeBu-MePy. The differences in attraction between the enantiomers of 2-MeBu-MePy appear to be more pronounced than for 2-MeBu-diMePy, an observation also noted in Experiment 1.

Overall, *W. auropunctata* worker responses to lures were similar to those reported by Troyer et al. (2009). When attractive treatments were placed in a counting area, *W. auropunctata* workers generally began to respond by orienting toward the pheromone source within a few minutes of placement. All pyrazine lures appeared to increase ant locomotion compared to the normal pace of ants travelling along trails. A previously unnoted phenomenon was observed when septa loaded with (*S*)-2-MeBu-diMePy were introduced to the treatment area on tree branches. In several cases, most of the ants on the nearby trail were observed to become quiescent and would "freeze" in place on the trail for several minutes. The activity cessation seemed to begin from the center of the treatment site and expanded to the far ends of the ant trail in seconds. Ants appeared to resume normal levels of activity on the trail after either the pyrazine was removed for a period of up to 30 min, or a disturbance was introduced, such as gently blowing air over the ants.

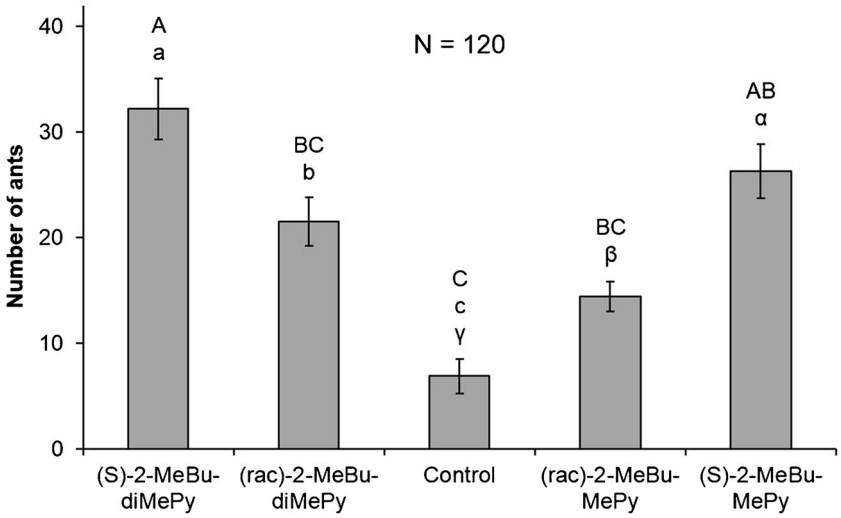

Fig. 3 Experiment 1. Numbers (mean±SE) of *Wasmannia auropunctata* counted in defined area for each treatment at 5-min intervals (*N*=total number of observations; bars with different letters are significantly different ($P<0.05$) (ANOVA, followed by Tukey's HSD); capital letters represent significant difference between all treatments, while significant difference between pyrazine treatments is shown in lower case (2-MeBu-diMePy) and Greek (2-MeBu-MePy) letters

Discussion

The identified *W. auropunctata* alarm pheromones are chiral due to the 2-methylbutyl moiety attached to the pyrazine. As chirality of ant pheromones has been reported to affect the behavior of these social insects (Morgan 2009), we were interested to determine if *W. auropunctata* workers responded differently to the enantiomers of 2-MeBu-diMePy and 2-MeBu-MePy. However, given the previous inability to resolve the enantiomers of both these alarm pheromones using enantioselective GC columns, it was difficult to determine the naturally occurring enantiomeric composition of the pheromone. This necessitated the synthesis of both enantiomers to facilitate behavioral work.

Synthesis While the (*S*)-enantiomers of 2-MeBu-diMePy and 2-MeBu-MePy are readily prepared from commercially available (*S*)-2-methyl-1-butanol, the corresponding (*R*)-alcohol is not readily available and its synthesis is not trivial (Sasaerila et al. 2000). The PCL-catalyzed kinetic resolution is a simple, metal free and environmentally friendly method for the preparation of this chiral intermediate. Although preparation of highly enantiopure (*R*)-**3** required the consumption of most of the racemic starting material, using succinic anhydride as an acylating agent made the separation of (*R*)-**3** from the reaction mixture a simple acid–base extraction.

We found that it is not necessary to generate the diisopentylzinc reagent *in situ* to induce cross-coupling with the chloropyrazine as previously reported (Sato and Matsuura 1996). In the previous case, pyrazines were coupled with diisopentylzinc that was generated *in situ* from isopentylmagnesium bromide and zinc bromide with 70 % yield. By not making the alkylzinc reagent, the risk of introducing water into the reaction system was substantially lowered as zinc bromide is highly hygroscopic. In the current synthesis, the coupling of chloropyrazine with Grignard reagent catalyzed by dichloro[1,3-bis(diphenylphosphino)propane] nickel(II) gave yields higher than 80 %.

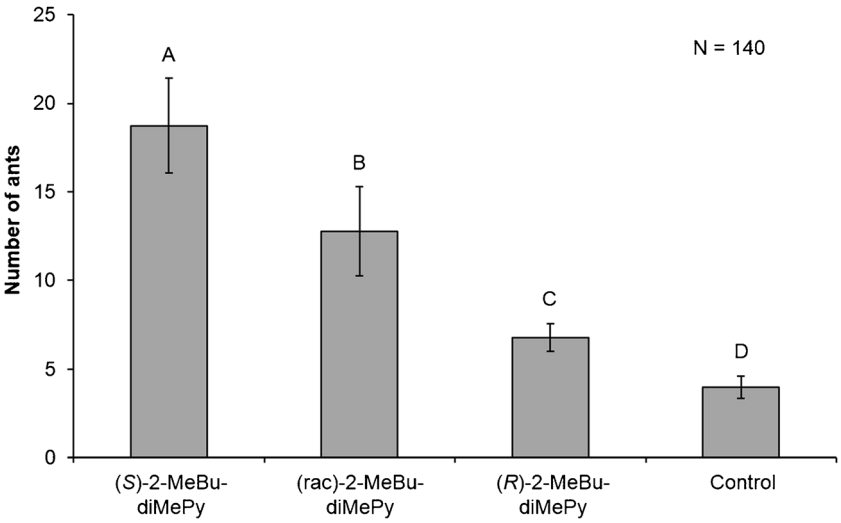

Fig. 4 Experiment 2. Numbers (mean±SE) of *Wasmannia auropunctata* counted in defined area for each treatment at 4-min intervals (*N*=total number of observations; bars with different letters represent significant difference ($P<0.05$) between treatments (ANOVA, followed by Tukey's HSD)

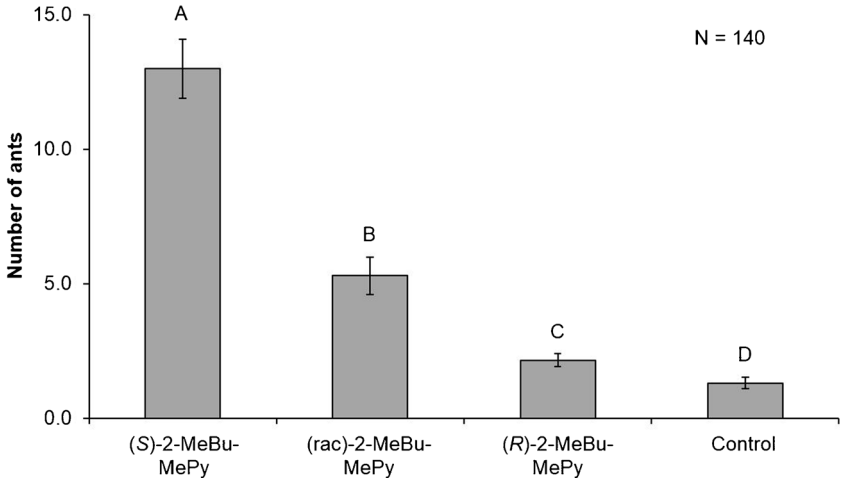

Fig. 5 Experiment 3. Numbers (mean±SE) of *Wasmannia auropunctata* counted in defined area for each treatment at 4-min intervals (*N*=total number of observations; bars with different letters represent significant difference ($P<0.05$) between treatments (ANOVA, followed by Tukey's HSD)

Stereochemical Analyses The enantiomers of both 2-MeBu-diMePy (**1**) and 2-MeBu-MePy (**2**) could be separated on a γDEX120 column. The γDEX120 column was designed to resolve medium to large size molecules, so it was not surprising that 2-methyl-1-butanol did not resolve on this column, while the enantiomers of **1** were resolved with a long run time (about 60 min, baseline separation). Further, the monomethyl enantiomers of **2** were resolved with an extremely long run time (about 115 min) and still without base-line separation, which may go some way to explain our previous failure to resolve enantiomers of **1** and **2** using Rt-βDEXm and Lipodex-E chiral columns. Using the γDEX120 column we were able for the first time to determine that the natural pyrazines produced by *W. auropunctata* are exclusively the (*S*)-enantiomers.

The discrepancy in ee values between intermediate **3** and coupling product **1** was likely due to the small amount of (*S*)-enantiomer in the resolved (*R*)-enantiomer sample of intermediate **3** being underestimated while using the Rt-βDEXm column. The Rt-βDEXm column did not provide baseline separation of the two enantiomers. Unfortunately, the γDEX120 chiral column also failed to resolve intermediate **3**. The racemization of the Grignard reagent of **3** was unlikely as the stereocenter is β from the free radical site.

Field Bioassays Chiral insect semiochemicals are not uncommon (Mori 2007). However, many of the pyrazine trail pheromones identified among ants have no stereocenter, and therefore, no chiral selectivity is seen (Morgan 2009). Chiral 2-MeBu-diMePy and 2-MeBu-MePy, therefore, provided us a chance to study the effect of chirality on the behavior of this invasive ant species. The first experiment (Fig. 3), conducted with the most readily-prepared (*S*)-enantiomers, showed the pure enantiomers were significantly more attractive than their corresponding racemate, and both racemates of 2-MeBu-diMePy and 2-MeBu-MePy were significantly more attractive than control. The lower activity of the racemates suggested that the (*R*)-enantiomer is less attractive but not repellent, because both racemates and (*S*)-enantiomers were prepared at the same concentration, such that the racemic lures contained half the dose of the pure (*S*)-enantiomer compared to the lures containing the pure enantiomers.

When both enantiomers were tested in field Experiments 2 and 3, (rac)-2-MeBu-diMePy was significantly less attractive than the (*S*)-enantiomer but more attractive than (*R*)-enantiomer, thus clearly demonstrating that *W. auropunctata* were able to distinguish both isomers. A similar trend was observed with the minor alarm pheromone component, 2-MeBu-MePy. These results are consistent with the fact that only (*S*)-2-MeBu-diMePy (**1**) and (*S*)-2-MeBu-MePy (**2**) were produced by the ants, although it is not always the case that the naturally occurring enantiomers are the most active (Mori 2007).

Given that all (*R*)-enantiomers used in the field bioassays contained trace amounts of the (*S*)-enantiomers, it seems reasonable to assume that some or all of the behavioral activity of these lures should be attributed to the (*S*) contaminants. Previous field worked by Troyer et al. (2009) showed that as little as 10 μg of 2-MeBu-diMePy attracted more *W. auropunctata* workers than unbaited controls. It was hence not possible to determine whether the (*R*)-enantiomers have any behavioral activity.

The difference in the number of ants attracted between (*S*)-2-MeBu-diMePy and (*R*)-2-MeBu-diMePy was less pronounced than that between (*S*)-2-MeBu-MePy and (*R*)-2-MeBu-MePy. The (*R*)-enantiomers of both 2-MeBu-diMePy and 2-MeBu-MePy were synthesized from the same intermediate using a Kumada coupling reaction (Kumada et al. 1978) employing a Grignard reagent. The latter can sometimes epimerize at the α carbon, due to the radical intermediate, but not at the β carbon stereocenter as in the 2-methylbutyl moiety (Kumada et al. 1978). Even if the resolution did not provide 100 % enantiomeric excess in the intermediate alcohol, both pyrazines should have contained the same ratio of enantiomers, suggesting that the differences in relative

behavioral responses are not attributable to ee differences. This might indicate that the pheromone detecting system of *W. auropunctata* is less sensitive to enantiomers of 2-MeBu-diMePy than to that of 2-MeBu-MePy. Alternatively, mixtures of (*R*)- and (*S*)-2-MeBu-MePy may not be additive, as they appear to be for 2-MeBu-diMePy.

We have previously have used traps baited with (*S*)-2-MeBu-diMePy and found them to be a highly species-specific detection method for *W. auropunctata* (Derstine et al. 2012). However, at that time it was not known if the (*S*)-enantiomer was more attractive than the racemic mixture, or which enantiomer is produced by the ants (Derstine et al. 2012). With the discovery of the enantiomeric preferences of *W. auropunctata*, use of (*S*)-2-MeBu-diMePy can now be confirmed as the preferred attractant for trapping.

Alarm pheromones may elicit aggressive responses, such as increased linear movement, increased sinuosity of movement, biting, extrusion of the sting, and recruitment, *etc.* (Parry and Morgan 1979; Vander Meer and Alonso 1998). Olubajo et al. (1980) previously reported that 4-heptanone, 4-heptanol, or a mixture of these chemicals elicited a non-aggressive response in *Zacryptocerus varians* where the ants stopped moving and backed up. The phenomenon we observed, that the whole section of ants on the visible ant trail stopped moving and the trail flow paused for minutes when (*S*)-2-MeBu-diMePy was applied, may be similar to that by Olubajo et al. (1980). We previously noticed an increase in *W. auropunctata* locomotion when exposed to 2-MeBu-diMePy and 2-MeBu-MePy (Showalter et al. 2010; Troyer et al. 2009). The contrast between such behavioral responses might indicate that (*S*)-2-MeBu-diMePy has both attractant and inhibitory effects depended on the doses. It is possible that "freezing" *W. auropunctata* workers may make them more vulnerable to sprayed pesticide, or competitor ants, predators, or parasitoids.

In light of the behavioral importance of chirality in *W. auropunctata* responses to pyrazine alarm pheromone enantiomers, it may prove interesting to investigate the role chirality could play in other ants known to produce alkylpyrazines. In particular, six other ant species, *Dinoponera australis* Emery (Oldham et al. 1994), *Odontomachus bauri* Emery (Morgan et al. 1999), *Rhytidoponera metallica* (Smith) (Tecle et al. 1987), *Rhytidoponera victoriae* (Andre) (Brophy 1989), a *Calomyrmex* sp. (Brown and Moore 1979), and an *Ectatomma* sp. (Morgan et al. 1999), are known to secrete 2-MeBu-diMePy from mandibular glands. Additionally, in reviewing the literature on ant alkylpyrazines, we identified 14 other pyrazines, from an additional 5 ant species, for which absolute configurations are yet to be determined (Brophy 1989; Tentschert et al. 2000). It is probable that at least some of these alkylpyrazines are produced as enantiomerically pure or enriched isomers, and that some ants would be expected to display differential behavioral responses to these compounds.

In summary, we have determined that only (*S*)-enantiomers of 2-MeBu-diMePy and 2-MeBu-MePy are produced by *W. auropunctata* workers, and that (*S*)-enantiomers induce the most attractive responses. It is hoped that this information can lead to the development of a more effective species specific trapping detection/management system for *W. auropunctata*.

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