J Chem Ecol (2010) 36:163–170
DOI 10.1007/s10886-010-9743-0

Isolation of a Pyrazine Alarm Pheromone Component from the Fire Ant, *Solenopsis invicta*

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Received: 22 September 2009 / Revised: 16 December 2009 / Accepted: 7 January 2010 / Published online: 10 February 2010
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Abstract Alarm pheromones in social insects are an essential part of a complex of pheromone interactions that contribute to the maintenance of colony integrity and sociality. The alarm pheromones of ants were among the first examples of animal pheromones identified, primarily because of the large amount of chemical produced and the distinctive responses of ants to the pheromone. However, the alarm pheromone of the fire ant, *Solenopsis invicta*, eluded identification for over four decades. We identified 2-ethyl-3,6-dimethylpyrazine as an alarm pheromone component of *S. invicta*. Worker fire ants detect the pyrazine alarm pheromone at 30 pg/ml, which is comparable to alarm pheromone sensitivities reported for other ant species. The source of this alarm pheromone are the mandibular glands, which, in fire ants, are not well developed and contain only about 300 pg of the compound, much less than the microgram quantities of alarm pheromones reported for several other ant species. Female and male sexuals and workers produce the pyrazine, which suggests that it may be involved in fire ant mating flight initiation, as well as the typical worker alarm response. This is the first report of 2-ethyl-3,6-dimethylpyrazine from a *Solenopsis* species and the first example of this alkaloid functioning as an alarm pheromone.

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Keywords Alarm pheromone · Pyrazine · Mandibular gland · Fire ant

Introduction

In social insects, maintenance of colony cohesiveness, sociality, and defense depends on sophisticated pheromonal communication. Worker/worker chemical interactions include recruitment, colony immigration, alarm, and nestmate recognition. Male and female alate sexuals use pheromones to induce mating flights and mediate mating behaviors. Workers are attracted to queen-produced releaser pheromones that are related to queen grooming, feeding and care of eggs (Vander Meer and Alonso 1998). Queens also release primer pheromones that influence sexual competition, directly or through the workers (Vargo 1998; Vander Meer and Alonso 2002). Pheromone diversity in social insects is matched by the diversity of their exocrine glands. Sixty three different glands have been described for social insects; in ants (Formicidae), alone, there are at least 39 described exocrine glands (Billen and Morgan 1998).

Alarm pheromones are a major class of social insect releaser pheromone, which have no direct benefit to the recipient but serve to put other colony members in a high state of alertness. In response to alarm pheromone, a variety of alarm behaviors have been described, including rapid running, attraction to the source, colony dispersal, and aggressive postures (Vander Meer and Alonso 1998). The chemistry of ant alarm pheromones is diverse, but components typically have a molecular weight of 100–200 and have 5–10 carbons. The low molecular weight, and consequent high volatility, of alarm pheromones is necessary for a quick and highly transient information transfer. Alarm pheromones can be terpenoids, alcohols, aldehydes,

ketones, esters, nitrogen heterocycles, sulfur-containing compounds, or other types of molecules (Hölldobler and Wilson 1990; Billen and Morgan 1998).

The highly evolved fire ant, *Solenopsis invicta* Buren, uses a complex of pheromones, including a recruitment pheromone (Vander Meer 1986) and a queen-produced recognition pheromone (Glancey et al. 1984). Decades ago, Wilson (1962) defined alarm behavior in fire ants as the rapid, erratic movement of workers toward a disturbed worker, and suggested that the source was from the head and, secondarily, from Dufour's gland components. Interestingly, alarm pheromones also are likely involved in fire ant mating flights. Fire ant mating flights are characterized by the opening of the normally closed nest tumulus, and frenzied, alarm-like, activity by workers (sterile), and male and female sexual alates, prior to the alates taking flight (Markin et al. 1971; Obin and Vander Meer 1994). The alarm-like behavior of workers and alate sexuals is mediated by a releaser pheromone linked to the mandibular gland (Obin and Vander Meer 1994; Alonso and Vander Meer 1997).

Elucidation of the structure(s) of the alarm pheromone of *S. invicta* has been a perplexing problem since it was first described over four decades ago (Wilson 1962). We present here the isolation, identification, and source of an alarm pheromone component from workers, and female and male sexual fire ants.

Methods and Materials

Source of S. invicta Colonies All colonies were monogyne and queenright (functional queen, brood, and workers), and derived from newly mated queens or from monogyne colonies collected from the Gainesville, FL, USA area, at least one year previously. Colonies were maintained as previously described (Banks et al. 1981), fed crickets three times a week, and had access to a test tube containing water and a test tube containing 10% aqueous sucrose solution.

General Alarm Bioassay Ten to 15 colonies were used for each bioassay, unless otherwise specified. At least 2 h prior to beginning an experiment, approximately 100–200 workers and some brood from each colony were added to small plastic containers with the inside walls coated with Fluon® to prevent escape. Additionally, a piece of red cellophane was placed over one end of the container to simulate dark conditions and to encourage the ants to move their brood and settle into a quiescent group; the experiment was not started if the worker ants had not settled into quiescent groups. All treatments and controls were placed in 20-ml scintillation vials. Just prior to the time of evaluation, the vial cap was removed and 3 ml of headspace were quickly drawn into a 5-ml plastic syringe (Henke-Sass Wolf Co.). A single syringe was used for each control and treatment. One milliliter of headspace air then was discharged over a group of quiescent ants, at a rate for which clean air (blank) would not generate a response, and the response of the ants observed and recorded. The response was evaluated on a scale of 1 to 4, with 1 = no response, 2 = some antennation, 3 = one to three workers running out of the resting group, and 4 = >3 workers running erratically from the resting group. Each evaluation lasted no longer than the length of time required to exhaust 1 ml of control air without alarming the ants. The experiment was conducted blind, with one person preparing the headspace sample and a second applying an unknown sample and reporting the response. All ant bioassay units were tested with all of the controls and treatments. Bioassay units were evaluated in sequence, but the samples given to the evaluator were randomized. In this way, a test unit that was alarmed had enough time to recover prior to the next test. To avoid complicating intra-colony responses, the workers in the bioassay units did not receive headspace samples from their own colony in the treatment/control vials. If negative or positive controls did not elicit the appropriate response, the experiment was terminated.

The *McNemar* test for significance of changes was used to analyze the alarm bioassay data. For analysis, bioassay scores of 1–2 were considered no reaction and scores of 3–4 were counted as alarm. The number of worker groups that displayed an alarm reaction to the test sample, but not to the negative control, was compared to the number of workers that reacted to the negative control but not to the test sample.

Shaken vs. Unshaken Workers Quiescent fire ant workers have an alarm response to headspace volatiles from shaken workers, but not to unshaken workers or clean air (Vander Meer et al. 2002). Unless specified otherwise, shaken and unshaken workers (100–200 in 20-ml glass scintillation vials coated on the upper inside surface with Fluon®) served as positive and negative controls, respectively. Air drawn from an empty 20-ml vial served as another negative control.

Amount of Venom Alkaloids in Shaken Ant Vials We tested whether shaking caused fire ant workers to release venom alkaloids. A thin layer of Fluon® was painted on the upper quarter of the inside surface of 20-ml scintillation vials, and about 100 fire ants were placed in each vial and weighed. The vials were shaken vigorously for 3–5 sec, and the ants were removed. *n*-Pentacosane (200 ng in 20 µl hexane) was added as an internal standard to the vial, which was then rinsed × 3 with hexane (100 µl each). The rinses were combined and the samples concentrated, if necessary, with

a gentle stream of nitrogen. A Varian 3700 GC, equipped with a flame ionization detector (FID) and a DB-1 fused silica column (0.32i.d.×30 m, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA), programmed from 150 to 285°C at 10°C min^{-1}, following a 2 min hold, was used for analysis. Injector and detector temperatures were 300°C. The data were analyzed with a Turbochome data analysis Workstation (Perkin-Elmer). Peak assignments were based on retention times of authentic *S. invicta* alkaloids, obtained by soaking workers in hexane overnight (see Ross et al. 1987). The total alkaloid quantity, relative to the internal standard, was determined This procedure was repeated five times.

Fire Ant Alarm Response to Venom Components Fire ant worker venom sacs were dissected (Vander Meer et al. 2002) and extracted in hexane at concentrations that approximated the amount found in vials from shaken ants (0.01 WE/10 μl hexane; WE = worker equivalent) and at 1 WE (per 10 μl hexane), used previously by Wilson (1962). Extracts (10 μl) were deposited on 1-cm square pieces of filter paper, the solvent allowed to evaporate, and the squares placed in 20-ml scintillation vials. The solvent control was prepared in the same way. Samples were evaluated in the alarm bioassay, as described above, along with shaken-ant positive controls.

Amount of Recruitment Pheromone in Shaken Ant Vials Shaken ants may release recruitment pheromone from their Dufour's glands, which could induce an alarm reaction in quiescent ants, as described by Wilson (1962). A sensitive recruitment orientation bioassay (modified from Barlin et al. 1976; Jouvenaz et al. 1978) was used to detect Dufour's gland products from shaken ants. This method utilizes a natural fire ant food trail that goes from the floor of the foraging area up a tongue depressor connected to two plastic platforms, the first platform used for the bioassay observation and the second containing food (crickets and 20% sucrose solution absorbed unto pieces of cotton). The base of each platform was coated with Fluon® to prevent ants from diverting their food trail down the sides of the platforms. A piece of paper (trail paper), the size of the platform, was placed on top of the first platform while the trail was developing. Pieces of paper (the same size as the platform) were marked with arcs on both sides, with both ends of the two arcs meeting. Ten microliters of treatment or control were applied to the two marked arcs of the paper (bioassay paper), and the solvent was allowed to evaporate. The bioassay paper was exchanged with the trail paper and the behavior of the ants observed. If at least one of the trailing ants followed the treatment trail from beginning to end, the test was scored positive; however, if the ants were confused at the two ends of the trail and no ants followed the test trail, the bioassay was scored negative. Due to the volatility of the recruitment pheromone, the bioassay was terminated after 2 min.

Three separate Dufour's gland extracts were prepared (Vander Meer et al. 2002) and diluted to a concentration of 0.1 WE/10 μl hexane. This concentration was subjected to bioassay and repeated dilution by a third until activity was lost. This allowed us to determine the approximate minimum Dufour's gland concentration needed for a positive bioassay. Extracts of shaken ant vials were prepared as described above, except no internal standard was added. Samples of these extracts were concentrated to 20 μl, with 10 μl used in the initial bioassay and the remaining 10 μl used for dilutions, if necessary. Responses of ants to these extracts were compared with responses of ants to the standard Dufour's gland dilutions, allowing determination of the approximate Dufour's gland concentration in WE.

Fire Ant Alarm Response to Recruitment Pheromone Dufour's glands were dissected as previously described (Vander Meer et al. 2002). Two concentrations were prepared: a) 1 WE/10 μl hexane, used by Wilson (1962) and b) 0.01 WE/10 μl hexane, the approximate amount released by shaken ants (see Results). Dufour's gland extract (10 μl) was deposited on a 1-cm square piece of filter paper, the solvent allowed to evaporate (ca. 20 sec), and the paper placed in a 20-ml scintillation vial. The solvent control was prepared in the same way. Samples were evaluated in the alarm bioassay as described above.

Solid Phase Micro Extraction (SPME) Collection Black (Carboxen/ Polydimethylsiloxane) SPME fibers (Supelco, Belefonte, PA, USA) were used to collect and desorb alarm pheromone volatiles for analysis. The fibers were thermally cleaned in the injection port of a GC for 2 h at 290°C or overnight at 280°C, prior to and after use. Approximately 1–1.2 g of worker ants were placed in a 20-ml glass scintillation vial, lined with Fluon® along the upper quarter to prevent escape. Each vial was sealed with a cap containing a small hole (large enough for insertion of the SPME needle) in the center. The ants were allowed to settle for approximately 1 h before headspace collection. For the unshaken ant treatment, a vial with worker ants was placed carefully under the SPME holder, and the fiber was lowered into the vial; volatiles were collected for 1 min. The SPME fiber then was retracted and the process repeated nine more times prior to desorption. For the shaken-ant treatment, a vial, containing worker ants, was shaken vigorously for 5 sec and then sampled by SPME, as described for unshaken ants.

Compounds absorbed onto the SPME fiber were analyzed initially on an Agilent 6890 GC, equipped with a FID (250°C), splitless injector (250°C), and a 30 m× 0.25 mm i.d. DB-23 capillary column. The column oven was

programmed from 40°C to 100°C at then 5°C min^{-1}, then to 250°C at 25°C min^{-1}. The SPME fiber was retracted 2.5 min. after insertion into the injector. SPME fibers also were analyzed by gas chromatography-mass spectrometry (GC-MS), using the same model GC and conditions, interfaced with an Agilent Mass Selective Detector 5973 (Palo Alto, CA) in the electron impact (EI) mode.

Identification of the Pyrazine Released by Shaken Fire Ant Workers The tentatively identified alarm pheromone component, 2-ethyl-3,5-dimethylpyrazine, was commercially available as a mixture with the 2-ethyl-3,6-dimethylpyrazine isomer (Aldrich Chemical Co, Milwaukee, WI, USA). Samples of these two isomers also were obtained (purified by preparative gas chromatography and 99% pure individually; Buttery and Ling 1997) from the laboratory of Dr R. M. Buttery. The identity of the natural alarm pheromone compound was established by co-injection and comparison of its retention time with those of the standards.

Synthetic chemicals were tested in the alarm bioassay at 1 ng/µl and 100 ng/µl in light mineral oil (Fisher Scientific, Fairlawn, NJ, USA). The oil did not elicit an alarm reaction and acted to slow the release of the volatile pyrazines. The negative controls for these tests were mineral oil, air, and unshaken ants. The positive control was the shaken ants. For all mineral oil bioassays, 1 ml of test material was placed in a 20-ml scintillation vial and tightly capped until use.

Quantification of Pyrazine Concentration in Headspace of Mineral Oil Standards Solutions of 100, 66, 33, 10, 6, and 3 pg/µl, in hexane, of 2-ethyl-3,6(3,5)-dimethylpyrazine were analyzed on the Agilent 6890 N-5973, in the Selective Ion Monitoring (SIM) mode, monitoring *m/z* 39, 42, 56, 81, 108, 135, and 136, in order to generate a standard curve. Since the standard consisted of two isomers, the ion abundances for each isomer were added to generate the standard curve. To determine synthetic pyrazine concentrations in the headspace of the mineral oil treatments used in the alarm bioassay, standards in light mineral oil, of concentration 100, 50, 25, 10, and 1 ng/µl, were prepared, and 1 ml of each was transferred to separate 20-ml scintillation vials. The headspace from each of these standards was used in the alarm bioassay, while another set of identically prepared standards was analyzed by GC-MS (sampling 100 µl of headspace) and used to determine pyrazine headspace concentration by comparison with the standard curve.

Qualitative Analysis of 2-Ethyl-3,6-Dimethylpyrazine in Mandibular Gland, Head and Thorax Quantities of the pyrazine in mandibular gland extracts from male and female alate sexuals and workers were determined. Female or male alate sexuals, or large workers, were collected in a scintillation vial with the upper quarter inner surface painted with Fluon®. The ants were allowed to become quiescent for at least 20 min and then frozen in a dry-ice acetone bath. An ant was placed on a watch glass, resting on ice under the microscope, with dorsal side up, and the top of the head punctured, and the cuticle layer was peeled away toward the front. The remaining (bottom) part of the head exoskeleton was held, and one of the mandibles was pulled gently. If the mandible disengaged from the associated musculature and there was no visible lumen, the sample was discarded. If the mandible was pulled out with some musculature and/or a visible lumen, it was transferred to a clean probe and placed in hexane in a vial insert. Twenty mandibles were accumulated per sample, and each sample was extracted in 50 µl hexane at 4°C overnight prior to analysis. An internal standard (IS), 2-methyl-3-ethyl pyrazine (100 pg), was added to the samples. Later, in order to shorten the time required for mandibular gland dissections, we dissected a minimum frontal part of the head that included mandibular glands, but excluded the postpharyngeal gland and antennae. Head and thorax samples (20 of each) of female and male alates, and workers also were collected and extracted as above.

Samples were analyzed by GC-MS using the DB-23 column, with helium as carrier gas. The GC oven was held at 40°C for 2 min, then increased to 150°C at 15°C min^{-1}, held for 5 min., then increased to 250°C at 10°C min^{-1}. Compounds were quantified by SIM, monitoring *m/z* 121 and 122 (for the IS) and 108, 135, and 136 (for 2-ethyl-3,6-dimethylpyrazine). Quantification of the alarm pheromone was achieved by comparing the total ion area of the pyrazine component to the total ion area of the IS. Analyses were carried out on workers (N=16), female alates (N=19), and male alates (N=10). The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) using Statview 5.0 software.

Results

Amount of Venom Alkaloids in Shaken Ant Vials The mean weight of ca. 100 worker ants was 73.3±4.4 mg (mean ± SE, N=5). The mean weight of venom alkaloids released by the ants onto the vial after being shaken was 211.0±48.6 ng per vial (mean ± SE, N=5). Each venom sac contains ca. 30 µg of total alkaloid (Vander Meer 1988); therefore, the shaken ants deposited only 0.007 poison sac equivalents onto the glass vial.

Amount of Recruitment Pheromone in Shaken Ant Vials The lowest detectable Dufour's Gland equivalent from standard Dufour's gland extracts in the bioassay was 0.0033±0.0013 WE (mean ± SE, N=3). This represents

approximately 1.3±0.8 pg of *Z,E*-α-farnesene per cm of trail, (mean ± SE, *N*=3), since a worker Dufour's gland contains approximately 4 ng of *Z,E*-α-farnesene (RVM, unpublished). Recruitment orientation bioassays with residues in the vials from shaken ants showed no activity in 2 of 3 replicates. The active replicate showed minimal activity (one ant completely followed the trail within the 2 min. time limit of the bioassay), and activity was lost with the next serial dilution. Therefore, shaken ants probably release extremely low levels of recruitment pheromone, between 0.0067 and 0.0033 WE of a Dufour's gland.

Fire Ant Alarm Response to Poison Sac and Dufour's Gland Components Headspace from 1 WE poison sac samples elicited a lower alarm response in fire ant workers than the shaken ant positive control (*G*=4.167, *P*=0.041, *df*=1, *N*=15), but elicited a significantly greater response than the air negative control (*G*=6.125, *P*=0.013, *df*=1, *N*= 15) (Fig. 1). The alarm response from the lower poison sac concentration (0.01 WE) was not different from the air negative control (*G*=0.8, *P*=0.371, *df*=1, *N*=15) (Fig. 1). One WE of poison sac can generate an alarm response in fire ants; however, the small amount of poison sac contents released by shaken ants did not contribute significantly to the observed alarm response induced by the headspace above shaken ants (Fig. 1). Dufour's gland extracts at 1.0 WE gave an alarm response that was not different from the alarm response from shaken ants (*G*=1.333, *P*=0.248, *df*= 1; *N*=14). When the concentration was reduced to 0.01 WE of a Dufour's gland, the alarm response was not different from that of the negative air control (*G*=0.000, *P*=1.000, *df*=1; *N*=14). The alarm response of the 1.0 WE Dufour's gland concentration was greater than the response of the 0.01 WE concentration (*G*=8.100, *P*=0.004, *df*=1; *N*=14). As shaken fire ant workers release less than 0.01 WE of Dufour's gland products, these results suggest that the alarm reaction from shaken ants is not due to the release of Dufour's gland products.

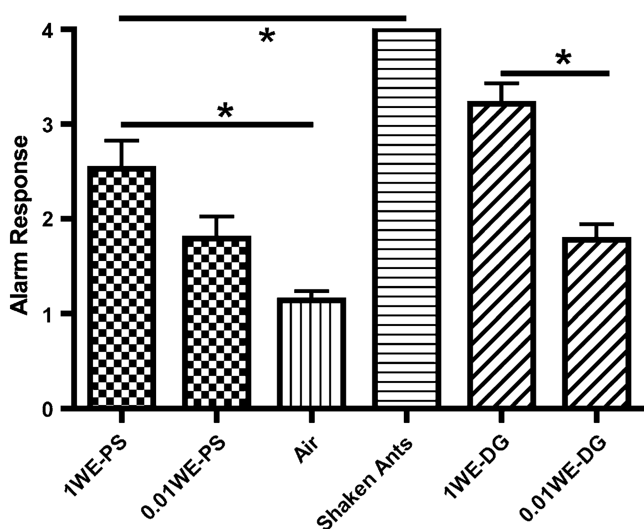
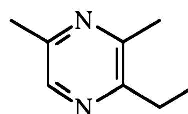


Fig. 1 Fire ant worker alarm responses to the headspace above, two poison sac extract concentrations (*WE* worker equivalent), two Dufour's gland (*DG*) extract concentrations, a positive control (shaken ants), and a negative control (air). The mean + SEM are shown (*N*= 15). An * between two columns indicates a statistical difference ($P<0.05$ *McNemar*'s Test)



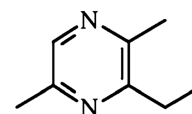


Fig. 2 Structures of the two pyrazine isomers, 2-ethyl-3,6-dimethylpyrazine and 2,6-dimethyl-3-ethylpyrazine

Analysis of SPME Absorbed Volatiles Comparison of GC profiles from quiescent and shaken workers showed a difference of one major compound. The mass spectrum of this peak had a fragmentation pattern that matched 2-ethyl-3,5-dimethylpyrazine (2,6-dimethyl-3-ethylpyrazine; NIST 98 Mass Spectral Library, Rev.D.02.00, Palo Alto, CA, USA). Gas chromatographic analysis of the standards of this compound and the 2-ethyl-3,6-dimethylpyrazine isomer (Fig. 2), on a polar column, showed that the 2-ethyl-3,6-dimethylpyrazine eluted first (Buttery and Ling 1997). Our GC analysis showed that the natural compound co-eluted with this compound.

Alarm Pheromone Bioassay of 2-Ethyl-3,6-Dimethylpyrazine and 2-Ethyl-3,5-Dimethylpyrazine The alarm bioassay results for the commercial mixture of 2-ethyl-3,5(3,6)-dimethylpyrazine (ca. 60% 3,5-dimethyl isomer and 40% 3,6-dimethyl isomer) and the individual isomers are shown in Fig. 3. Responses to 1 ng/µl 2-ethyl-3,6-dimethylpyrazine were not significantly different (*G*=3.20, *df*=1, *P*=0.074, *N*=15) from the responses to the positive control of shaken ants, but were significantly greater (*G*=6.125, *df*=1, *P*= 0.013, *N*=15) than those to the negative control of unshaken ants. In contrast, the alarm response to the 1 ng/µl synthetic mixture of 2-ethyl-3,5(3,6)-dimethylpyrazine was less (*G*= 6.125, *df*=1, *P*=0.013, *N*=15) than that to the shaken ant positive control, but not different from that to the negative control of unshaken ants (*G*=3.2, *df*=1, *P*=0.074, *N*=15). By inference, the alarm response induced by the headspace above 1 ng/µl 2-ethyl-3,5-dimethylpyrazine was significantly less than that induced by the shaken ants, and not different from that by the unshaken ants. Responses to all 100 ng/µl treatment samples were not significantly different from the shaken ant positive control. In summary, 2-ethyl-3,6-dimethylpyrazine, had a lower active alarm response threshold than the 2-ethyl-3,5-dimethylpyrazine isomer and the mixture of the two isomers.

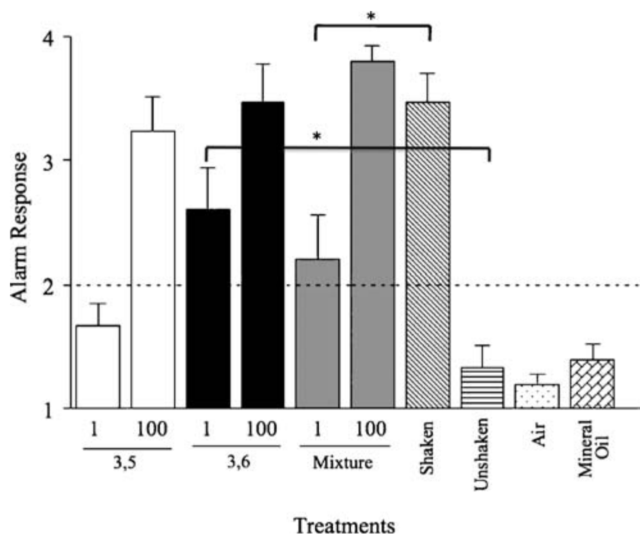


Fig. 3 Alarm responses of worker fire ants to two concentrations (in mineral oil) each of 2-ethyl-3,5-dimethylpyrazine (3,5), 2-ethyl-3,6-dimethylpyrazine (3,6), and a commercially available mixture of the two isomers (Mixture), negative controls (unshaken ants, air and mineral oil), and a positive (shaken ants) control. An * between two columns indicates a statistical difference ($P<0.05$, *McNemar*'s Test). An alarm response below the dashed line represents no alarm and alarm responses above the dashed line represents an alarm reaction

Alarm Response to Pyrazine Alarm Pheromone Concentration The fire ant alarm response to the headspace from a series of concentrations of the synthetic mixture of 2-ethyl-3,6(3,5)-dimethylpyrazine is shown in Fig. 4. The alarm response elicited from the headspace above the 1 ng/µl mixture was greater than that to the headspace above unshaken ants ($G=4.900$, $df=1$, $P=0.027$, $N=15$), but not different from that to headspace of the positive shaken ant control ($G=3.200$, $df=1$, $P=0.074$, $N=15$). By inference, all concentrations of the synthetic mixture of 2-ethyl-3,6(3,5)-dimethylpyrazine evaluated produced a greater alarm response than volatiles from the headspace above unshaken ants, with the exception of the lowest concentration, 100 pg/µl ($G=0.167$, $df=1$, $P=0.683$, $N=15$).

By using the standard curve ($y=1.3*10^6\times-628$; $R^2=0.9974$), derived from GC analysis of concentrations of the synthetic pyrazine mixture in hexane, the headspace concentrations of 100, 50, 25, 10, 1, and 0.1 ng/µl of the 2-ethyl-3,6(3,5)-dimethylpyrazine mixture in light mineral oil were determined as 0.5, 0.36, 0.24, 0.1, 0.03, and not detectable, pg/µl, respectively.

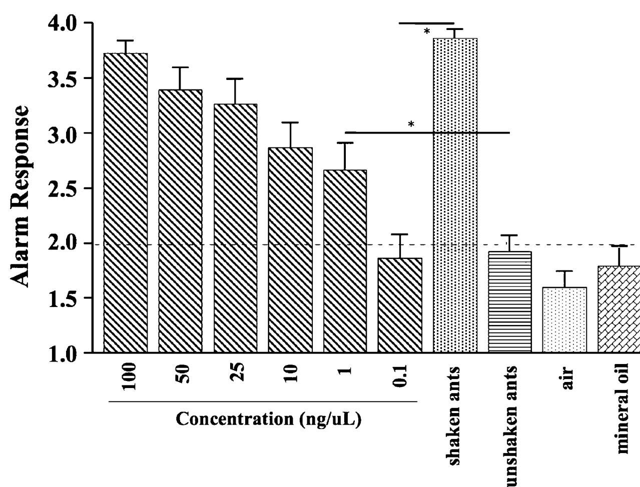


Fig. 4 Fire ant worker alarm responses to headspace above different concentrations of 2-ethyl-3,5(3,6)-pyrazine (in light mineral oil), negative controls (unshaken ants, air and mineral oil), and a positive (shaken ants) control. An * between two columns indicates a statistical difference ($P<0.05$, *McNemar*'s Test). An alarm response below the dashed line represents no alarm and alarm responses above the dashed line represents an alarm reaction

Pyrazine Alarm Pheromone in Adult Fire Ants Extracts of female and male alate and worker heads, and mandibles plus mandibular glands, all contained detectable amounts of 2-ethyl-3,6-dimethylpyrazine. In contrast, this compound was undetectable in thoracic extracts from these insects, demonstrating its specificity to the mandibular glands.

Workers contained significantly less 2-ethyl-3,6-dimethylpyrazine (316.6±42 pg/ant, $N=16$) than either female (746.9±80.8 pg/ant, $N=19$) or male (959.4±215.3 pg/ant, $N=10$) alates (Fisher PLSD, ANOVA, $P=0.002$ and $P<0.001$, respectively). The amounts of 2-ethyl-3,6-dimethylpyrazine in male and female alates were not significantly different (Fisher PLSD, ANOVA, $P=0.197$) (Fig. 5).

Discussion

Ant alarm pheromones are biosynthesized in a variety of exocrine glands and consist of chemicals of diverse

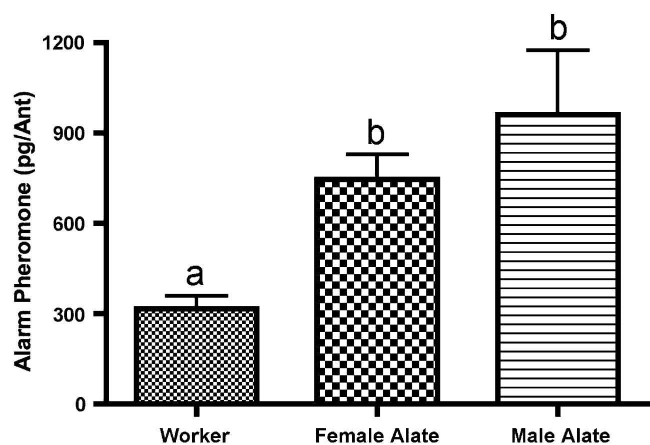


Fig. 5 The amount (mean + SEM) of 2-ethyl-3,6-dimethylpyrazine in fire ant workers (sterile females, $N=16$), and female ($N=19$) and male ($N=10$) sexual alates. Different letters above columns indicates means that are statistically different (*Fisher PLSD, ANOVA*, $P<0.05$)

structures (Vander Meer and Alonso 1998). Our identification of 2-ethyl-3,6-dimethylpyrazine as an alarm pheromone component of *S. invicta* increases the known diversity of ant alarm pheromones.

Pyrazines have been reported to have a variety of functions, including trailing (recruitment), defense, and alarm (El-Sayed 2009). The pyrazine reported here, 2-ethyl-3,6-dimethylpyrazine, has been reported primarily from the ant Subfamily Myrmicinae, which includes the genus *Solenopsis*, and also from the Ectatomminae, Dolichoderinae, and Ponerinae. In all cases, the function attributed to 2-ethyl-3,6-dimethylpyrazine is trailing (recruitment). While there are reports of 2-ethyl-3,6-dimethylpyrazine in mandibular glands of ants, no function has been attributed previously to this compound in ants (Cavill et al. 1984; Morgan et al. 1999). Another isomer, 2-ethyl-3,5-dimethylpyrazine, however, has been reported to be a part of the alarm pheromone of *Odontomachus brunneus* (Longhurst et al. 1978). This is the first report of 2-ethyl-3,6-dimethylpyrazine from a *Solenopsis* species, and is the first report of this alkaloid functioning as an ant alarm pheromone.

Although responses of fire ants to alarm pheromone previously have been documented (Wilson 1962), and the primary source of the pheromone known to be a cephalic gland, with attraction derived from a combination of the cephalic gland and Dufour's gland (Wilson 1962), the chemical identification of the fire ant alarm pheromone has eluded scientists. Our previous work had demonstrated that excited fire ant female and male sexual alates produce an alarm reaction in workers and that mandibular glands were the source of the alarm pheromone (Alonso and Vander Meer 1997). The ephemeral nature of fire ant alarm pheromones was demonstrated when headspace taken immediately after worker ants in a vial were electrically stimulated, elicited an alarm reaction. However, leaving the vial open for 2 min resulted in no alarm reaction (Vander Meer et al. 2002). This also partly explains the inability of some researchers to verify a cephalic source for fire ant alarm pheromones (Blum 1980).

Shaking a vial of worker ants revealed that both Dufour's gland and poison sac contents were deposited onto the vial, but at an amount less than one hundredth of a gland equivalent. This concentration did not elicit an alarm response from worker ants, although one Dufour's or poison gland equivalent did produce a significant alarm reaction. These results support the early alarm pheromone report by Wilson (1962), who used crushed worker body parts (one WE) to determine that the head, and probably the Dufour's gland, induced fire ant alarm responses. However, our results indicate that at physiologically relevant levels, neither Dufour's gland nor poison gland products are involved in eliciting the natural alarm response in fire ants, and that fire ant mandibular glands are the source of the alarm pheromone.

Because of the difficulty in obtaining behaviorally active extracts from worker mandibular glands, we used SPME to compare headspace above quiet and shaken workers to identify 2-ethyl-3,6-dimethylpyrazine as a unique component released from shaken fire ant workers. We then were able to detect and quantify this compound in the mandibular glands of workers, and male and female sexual alates. Our results showed that workers contain only about 300 pg of the pyrazine, which is consistent with the morphological description of fire ant mandibular glands as small and comprised of only a few secretory cells (Phillips and Vinson 1980; Billen 1990). The small quantity of alarm pheromone from fire ant workers is in sharp contrast to the microgram quantities (>3,000 fold more) of alarm pheromone found in other ant species, e.g., *Lasius alienus* Dufour's gland contains microgram quantities of alarm pheromone (Regnier and Wilson 1969). The high volatility of the fire ant pyrazine and the small quantity produced explains why isolation and identification of the alarm pheromone of *S. invicta* had proven difficult.

Fire ants respond to the pyrazine mixture at a concentration of 30 pg/cm^3, or 1.3×10^{11} molecules/cm^3, which is comparable to the alarm sensitivity of 10^{10}–10^{13} molecules/cm^3 predicted by a model (Regnier and Wilson 1968), and to alarm threshold measurements of $>10^{11}$ molecules/cm^3 for *Pogonomyrmex badius* (Wilson 1958). The detection threshold of 2-ethyl-3,6-dimethylpyrazine by humans is extremely low, roughly 3.8×10^{13} molecules/cm^3 (Buttery and Ling 1997). In comparison, the fire ant is about 300 times more sensitive to this compound.

Ants produce a wide variety of alkaloids (see El-Sayed 2009). Fire ants produce a variety of 2-methyl-6-alkyl or alkenylpiperidines (MacConnell et al. 1971) that are used by workers in a variety of defensive contexts (Obin and Vander Meer 1985) and for prey procurement. These alkaloids are produced by the poison gland and stored in large quantities in the venom sac (Vander Meer 1988) and have a variety of physiological functions e.g., insecticidal, antimicrobial, and herbicidal (Obin and Vander Meer 1985; Escoubas and Blum 1990). The patterns of piperidine alkaloid homologues have been shown to be species-specific and are useful taxonomic tools (Brand 1978; Vander Meer and Lofgren 1988). Our identification of a pyrazine alarm pheromone component, produced by mandibular glands of fire ants, adds to our knowledge of the variety of alkaloid biosynthesis in glands of ants.

Future research will focus on the ontogeny of 2-ethyl-3,6-dimethylpyrazine production in the fire ant, isolation and identification of additional fire ant alarm pheromone components, and the species specificity of 2-ethyl-3,6-dimethylpyrazine.

Acknowledgements We thank M. Custer, T. Krueger, and D. Milne for technical support, and Drs. S Allan and S. D. Porter for critically reviewing the manuscript. We are grateful to Dr. Buttery for graciously providing synthetic samples of the two pyrazines.

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