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ISOLATION OF THE TRAIL RECRUITMENT PHEROMONE OF *Solenopsis invicta*

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Abstract—The *Solenopsis invicta* trail pheromone is synthesized by the Dufour's gland and is released through the sting apparatus. The recruitment subcategory of the *S. invicta* trail pheromone was shown to be composed of a mixture of the orientation pheromone, (*Z*, *E*)-α-farnesene and an unidentified homosesquiterpene consisting of three rings and one double bond (C−1). C−1 is present in worker Dufour's glands at only 75 pg per worker equivalent. This is the first report that demonstrates that different exocrine products from the same gland control different subcategories of behavior related to mass recruitment.

Key Words—*Solenopsis invicta*, Hymenoptera, Formicidae, imported fire ant, Dufour's gland, trail pheromone, pheromone, (*Z*, *E*)-α-farnesene, sesquiterpene.

INTRODUCTION

Ant trail pheromones represent one of the most elaborate known forms of chemical communication in social insects (Wilson, 1971) and were discussed in recent reviews of the behavior and chemistry of mass recruitment (see Hölldobler, 1978; Attygalle and Morgan, 1985). Trail pheromones are essential components of efficient foraging and mass recruitment by *Solenopsis invicta* Buren (Wilson 1962b) and are a good example of trail pheromone complexity. Wilson (1959) reported that the Dufour's gland was the source of fire ant trail phero-

mones and that the contents of the gland were emitted through the sting onto the substrate on which the trail-laying worker walks. The pheromone attracts workers who then orient (move) along the trail (Wilson, 1962a). Depending on the context, the Dufour's gland contents also released colony emigration, alarm recruitment, or settling behavior (Wilson, 1962c).

The investigation of the chemical nature of fire ant trail pheromones began with Wilson (1959), who recovered bioassay active material from a steam distillate of whole ants. However, the crude material was not stable over long periods of time (Walsh et al., 1965). Barlin et al. (1976) characterized the trail pheromone of *S. invicta* as a sesquiterpene hydrocarbon, based on analogy with gas chromatograph (GC) and GC-mass spectrometric (GC-MS) data for *Solenopsis richteri* Forel. This was confirmed by Vander Meer et al. (1981), who isolated (*Z,E*)- and (*E,E*)-3,7,11-trimethyl-1,3,6,10-dodecatetraene (α-farnesenes) and two homofarnesenes later identified as (*Z,E*)- and (*E,E*)-3,4,7,11-tetramethyl-1,3,6,10-dodecatetraene (Alvarez et al., 1987). An allofarnesene was also reported (Williams et al., 1981); however, we synthesized and tested this compound in our laboratory and showed that it did not induce trailing behavior (Williams, 1985).

The isolation of the farnesenes and homofarnesenes in the previous research was directed by a bioassay that specifically measured the orientation response of ants (movement along a trail). However, these compounds were ineffective in a bioassay designed to measure worker recruitment or attraction associated with the trail pheromone. We report here the isolation and partial identification of the Dufour's gland components from *S. invicta* that recruit workers to a trail.

METHODS AND MATERIALS

Point Source Recruitment Bioassay. Ten positions were marked symmetrically on the bottom of a plastic tray (7 × 44 × 56 cm) along a circle radiating 20 cm from the tray center. Queenright colonies consisting of one to three Williams nests (Banks et al., 1981) were reared from queens collected around Gainesville, Florida, and used in the bioassays. The cells were placed on top of each other in the center of the plastic tray. In addition, workers in the foraging arena of the mother colony tray were transferred to the bioassay tray. Each test colony contained approximately 25,000–75,000 workers. The ants were given at least 1 h to equilibrate in their new surroundings. Blotter paper squares (2 × 2 cm) with 1.5-cm-diam. circles marked in the center were placed on slightly larger aluminum squares. Up to eight test samples, a Dufour's gland extract standard, and a solvent blank (volume applied = 5 μl; one worker equivalent, WE) were applied to the paper squares and placed randomly on the numbered positions. The ants responded to active test samples by aggregating on the treated spots, often chewing the paper (see Wilson 1962c). The number of

ants aggregated within a 1.5-cm circle on the blotter paper squares were counted at 5-min intervals for 30 min. The sum of the six counts was regarded as the result for each position. Each test was replicated using six different *S. invicta* colonies. Experiments were evaluated by ranking sample responses based on Dufour's gland standard = 100 and solvent blank = 0. This method of scoring was used to minimize the day-to-day variability of the responsiveness of the ants. Using this method, if a sample response is greater than the Dufour's gland control, its ranking is greater than 100. Likewise if a sample's score is less than the solvent control, then its ranking will be less than 0.

Olfactometer Bioassay. The olfactometer (modeled after a design by Vander Meer et al., 1979) consisted of two 24/40 ground glass joints (Figure 1b); each ring was sealed to one of the arms of a 5-cm Y tube such that 1 cm of each Y-tube arm extended through the male half of one of the ground glass joints (Figure 1 d). A 5-cm piece of 0.6-cm-ID tubing was ring sealed 1 cm into the female half of the ground glass joints (Figure 1, a and e). A baffle (Figure 1c) at the center of the Y tube controlled air streams and prevented premature mixing of the sample, and gave the ants a clearer choice. The baffle also narrowed the openings to the choice chambers to the minimum size required

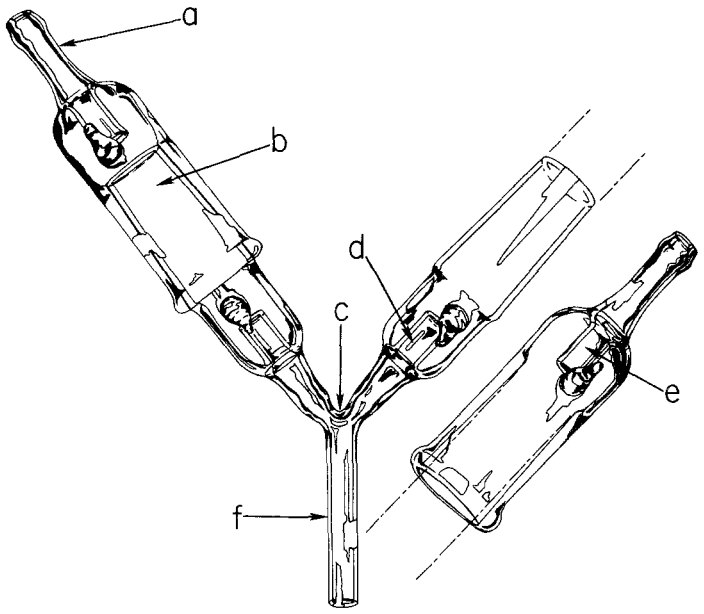

FIG. 1. Drawing of Y tube used in recruitment bioassays. Unit consists of two 24/40 ground glass joints ring-sealed to a Y stem. Legend: a, air inlet tube; b, sample chamber; c, baffle; d, ring seal tube (front); e, ring seal tube (rear); f, entrance stem.

for passage of a major worker. A test sample (10 μl/WE) and a solvent blank (10 μl) were applied to two filter paper strips (0.3 × 2.5 cm, Whatman No. 1), air dried, and each placed in one of the choice chambers (Figure 1, b). Compressed air (breathing air quality) was split into two streams and passed into the two choice chambers. Each stream was regulated to 0.2 liters/min. for a total effluent flow rate of 0.4 liters/min. Approximately 50–70 ants from laboratory colonies were confined in a 2.5-cm piece of 0.9-cm-ID Tygon tubing sealed at one end with wire gauze. The open end of the tubing was attached to the entrance stem (Figure 1, f). The initial choice of the first 20 ants that walked down the entrance tube and into one of the arms of the Y tube was recorded. Ants that were not trapped in a choice chamber and came back to the entrance stem were not counted if they made another choice. After each test, the olfactometer was rinsed with acetone and dried. Each test sample was retested with worker ants from the same colony, but the choice chamber in which the sample and control were placed was reversed. This procedure eliminated any bias inherent in the individual olfactometers. A complete replicate was the sum of the results from the two tests. Data were analyzed statistically by a chi-squared test, and comparisons between means were made with the Newman-Keuls test.

Gas Chromatography. Gas chromatography (GC) was performed on a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID). Small-scale preparative GC work was carried out using the FID and a 10:1 glass splitter. The effluent was collected in glass capillary tubes and eluted into vials with hexane. The GC columns used are designated as follows: Column A—DB-1 (J and W Scientific, Inc.), fused silica, 0.322 mm ID × 15 m, 0.25 μm film thickness; Column B—Superox (SGE, Inc.), glass, 0.5 mm ID × 27 m; Column C—OV-101 on 120/140 Gas Chrom Q (Applied Science), 4 mm ID × 1.8 m; Column D—3% OV-17 (Applied Science, Inc.) on Gas Chrom Q (120/140 mesh). Quantitative data were obtained with a Varian CDS 401 data processor and paraffin internal standards.

Dufour's Gland Extract. Dufour's glands were extirpated in water from randomly chosen worker ants and macerated in hexane. Pooled Dufour's gland extracts were used to determine parameters for recruitment pheromone isolation, GC quantification, and as standards in all bioassays.

Dufour's Gland Extract Column Chromatography. Dufour's gland extract (100 WE in 150 μl hexane) was applied to a 1.5-cm × 0.4-cm bed of silica gel (37–53 μm, Whatman, Inc.) and eluted sequentially with 6 ml each of hexane, methylene chloride, and methanol to separate the solute into hydrocarbon, nonpolar lipids, and polar lipids, respectively (Christie, 1973). The solvent for each fraction was evaporated under a stream of nitrogen to a volume of 0.5 ml and used in the point source recruitment bioassay.

Preparative GC of Dufour's Gland Extract. A Dufour's gland extract (100 WE) was separated into fractions by preparative gas chomatography (FID, 10:1

splitter, column C), 145°C for 22 min then 145–250°C at 10°C/min. Two procedures were used: (1) The mixture was separated into a volatile region (solvent front through the retention time of heptadecane) and a nonvolatile region (everything beyond heptadecane). (2) Individual fractions were collected at 1-min intervals for the first 21 min. Fractions 1–10 were from the solvent front to just after the elution of heptadecane. Fraction 22 contained all material eluted beyond 22 min. The fractions were collected in capillary tubes cooled with Dry Ice, and the sample was eluted into a 2-ml vial with 500 μl of hexane. These fractions were used for subsequent bioassays.

Isolation of Non-Dufour's Gland Saturated Hydrocarbons. The postpharyngeal gland (located in the head) is a rich source of species-specific hydrocarbons (Vander Meer et al., 1982). Five postpharyngeal glands were extirpated and crushed in 150 μl of hexane. This solution was applied to a Pasteur pipet silica column and eluted with hexane to isolate only the hydrocarbons (Christie, 1973). The hexane eluent was evaporated to a small volume and quantitatively analyzed by GC.

Extraction of Worker Ants. Whole worker ants (904 g, an estimated 904,000 workers at 1 mg per individual) were rinsed two times in hexane (1.5 liters) for 15 min and filtered. The ants were then homogenized in hexane (1.5 liters) for 2 min using a Virtis blender at a medium setting. The homogenate was filtered over a bed of Celite and the residue washed and filtered several times with fresh hexane. The filtrate was concentrated under vacuum to 200 ml. The insoluble residue was further homogenized with methanol (1.5 liters), filtered, washed several times with fresh methanol, and the combined filtrate was concentrated under vacuum to 200 ml. The hexane fraction was analyzed by thin-layer chromatography (TLC) using Polygram sil G/UV_{254} precoated plastic sheets (Macherey-Nagel, 40 × 80 mm) and an eluting solution for lipid separation (Christie, 1973).

Fractionation of Hexane Extract. The hexane extract (200 ml) was fractionated by gravity column chromatography on silica gel (800 g, Analtech; 35–75 μm). The sample was sequentially eluted with hexane (2 liters), methylene chloride (1.5 liter), and methanol (1 liter). These fractions were designated G-1, G-2, and G-3, respectively. The three fractions were concentrated under vacuum to 200 ml. The hexane eluent (G-1) was further concentrated under vacuum to yield 3.2 ml of a colorless liquid.

Separation of Saturated and Unsaturated Hydrocarbons. An aliquot (30 μl, 8500 WE) of the total hydrocarbon fraction (G-1) above was diluted in hexane (1 ml) and chromatographed using silver nitrate-impregnated silica gel (10% $AgNO_3$, Adsorbosil-LC-Ag, Applied Science, Inc., State College, Pennsylvania; 1.3 × 23 cm bed size). The sample was eluted stepwise with 0–50% ethyl ether in hexane (1 ml/min; 8.5 ml/fraction). Twenty fractions (Ag) were collected, and each was filtered through sodium chloride to remove potentially

interfering AgCl. The percentage of ethyl ether was increased based on GC analysis (column A) of fractions: fractions 1–6, hexane; fractions 7–11, 5% ethyl ether; fractions 12–16, 10% ethyl ether; fractions 17 and 18, 20% ethyl ether; and fractions 19 and 20, 50% ethyl ether. The fractions were designated Ag-1 to Ag-20.

Isolation of Active Components. Preparative GC of active fractions was carried out on columns C or D using an FID and 10:1 split. Kovats indices (KI) were calculated and coinjections made on columns A and B. Chemical ionization (C1) GC-MS were obtained on a Finnigan model 1015 SL upgraded to a 3200 GC-MS system. Hydrogenations (10% Pd/C in hexane) were carried out using a Supelco, Inc. microhydrogenator.

RESULTS

Preliminary studies using Dufour's gland extracts indicated that the active components of the recruitment pheromone were hexane soluble and could be eluted from a silica gel gravity column with hexane (Table 1). These physical properties defined the active components as hydrocarbons (Christie, 1973). Preparative GC of the Dufour's gland hexane extract yielded full bioassay activity from only the volatile region (solvent front through *n*-C-17 paraffin) of the chromatogram (Table 1). However, all pheromone activity was lost on preparative GC of the volatile region into 10 one-minute fractions. Recruitment pheromone activity was regenerated on recombination of all 10 fractions, which suggested a multicomponent pheromone system.

It was not practical to continue the elucidation of the recruitment trail pheromone complex using Dufour's gland extracts, since extirpation of adequate numbers of glands would be too labor intensive. Therefore, we extended the separation procedures developed for Dufour's gland extracts to the isolation of

TABLE 1. POINT SOURCE BIOASSAY OF COLUMN CHROMATOGRAPHY AND PREPARATIVE GAS CHROMATOGRAPHY FRACTIONS OF DUFOUR'S GLAND EXTRACTS

Sample[a]	Recruitment ranking[b]
A. Hexane	96.1 ± 15.5
B. Methylene chloride	29.5 ± 10.7
C. Methanol	9.9 ± 10.7
D. GC volatile region	164.0 ± 31.7
E. GC nonvolatile region	54.0 ± 15.3

[a] All samples were tested at 1 WE.
[b] Mean response (±SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

bioassay-active materials from whole ant extracts (Figure 2). Whole ants were first rinsed with hexane to remove cuticular hydrocarbons, which would add to the contamination of the hydrocarbon trail pheromone components. Then the ants were extracted sequentially with hexane and methanol. Analytical thin-layer chromatography of the hexane extract showed a complex mixture of substances with a range of polarities. Following the procedure used for Dufour's gland extracts, the whole ant hexane extract was separated into hydrocarbons, nonpolar lipids, and polar lipids (G-1 to 3) by sequential elution with hexane, methylene chloride, and methanol, respectively, from a silica gel column (Christie, 1973). Point source bioassays of the three fractions clearly showed that pheromone activity was only in the hexane fraction (G-1, Figure 2), which corresponded to hydrocarbons.

GC analysis of hydrocarbon fraction G-1 showed that the vast majority of hydrocarbons were the known saturated normal, methyl, and dimethyl branched

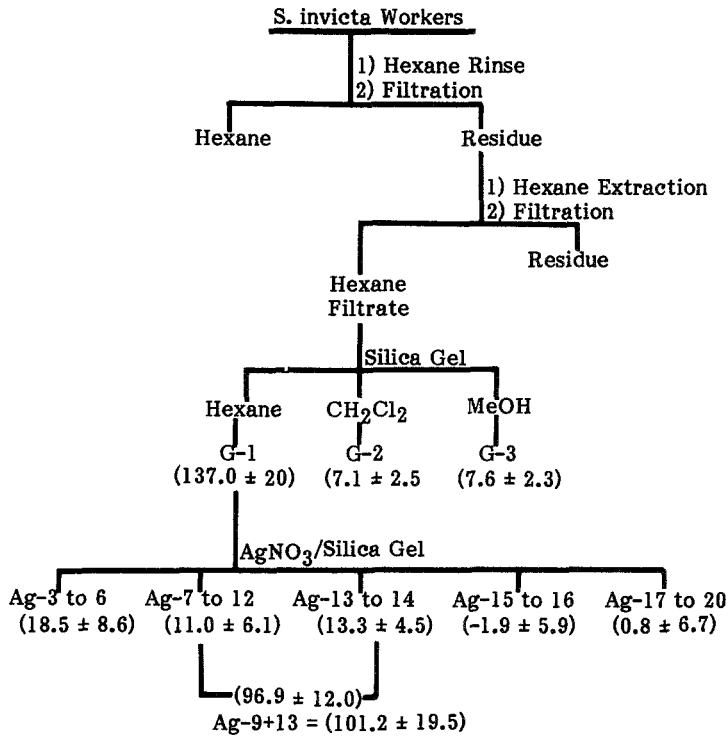

FIG. 2. Separation flow chart for the isolation of the *S. invicta* trail recruitment pheromone from worker extracts. Point source bioassay results are shown in parentheses (mean ± SE, $N = 6$) for the indicated fraction. Ranking is based on response to Dufour's gland = 100 and the response to the solvent blank = 0.

compounds specific to *S. invicta* (Lok et al., 1975; Nelson et al., 1980; Thompson et al., 1981). Our previous work on the trail orientation pheromone indicated that at least a part of the recruitment pheromone might be an unsaturated hydrocarbon. Consequently, to separate hydrocarbons with varying degrees of unsaturation, the total hydrocarbon fraction G-1 was chromatographed on a silver nitrate-impregnated silica gel column and eluted with hexane containing increasing amounts of diethyl ether. A total of 20 fractions were collected. The fractions were grouped into the following five categories based on changes observed in the GC chromatograms of each fraction: Ag-(3–6), Ag-(7–12), Ag-(13–14), Ag-(15–16), and Ag-(17–20). Point source bioassays of the five groups showed that none of the individually tested groups approached the activity induced by the Dufour's gland standard (Figure 2). However, bioassay results for various combinations of the five groups indicated that Ag-(7–12) in the presence of Ag-(13–14) gave full Dufour's gland activity (Figure 2). In addition, when these two groups were combined with the saturated hydrocarbon group (isolated in Ag-3, by GC analysis), activity greater than Dufour's gland was generated. These results led to detailed bioassay investigation of fractions within the Ag-(7–12) and Ag-(13–14) groupings. The combination of Ag-9 and -13 gave activity equivalent to that of Dufour's gland extracts, and these fractions were selected for preparative GC.

Ag-9 was initially separated into four major GC sections, Ag-(9A–D). Bioassays, always with an equivalent amount of Ag-13, indicated that only Ag-9C was active. Ag-9 was GC prepped again (Table 2), subdividing the active zone, Ag-9C, into four fractions, Ag-9(C-1 to C-4). The active area contained a single peak that was a minor component in Ag-9. This component was designated C-1. It was homogeneous on two capillary GC columns (KI 1640 on

TABLE 2. POINT SOURCE RECRUITMENT BIOASSAY RESULTS FOR PREPARATIVE GAS CHROMATOGRAPHY FRACTION Ag-9

Preparative GC fraction[a]	Recruitment ranking[b]
Ag-9A	26.3 ± 12.8
Ag-9B-1	9.7 ± 2.8
Ag-9B-2	11.4 ± 5.5
Ag-9C-1	130.1 ± 22.8
Ag-9C-2	12.8 ± 8.8
Ag-9C-3	21.0 ± 13.2
Ag-9C-4	30.6 ± 17.2

[a] All samples and Dufour's gland extracts were tested at 1 WE. All fractions were bioassayed with an equivalent amount of Ag-13.

[b] Mean response (± SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

column A and 1796 on column B). Knowing where to look on the GC chromatogram facilitated the discovery of C-1 in Dufour's gland extracts. Quantitative analysis of *S. invicta's* Dufour's glands (from a pooled sample of 100) showed that component C-1 was present at about 75 pg per worker. GC-MS of C-1 showed it to have a molecular weight of 218, indicative of a C_{16} hydrocarbon with four degrees of unsaturation (rings and/or double bonds). Hydrogenation followed by GC-MS showed the uptake of two hydrogens, which means that the compound has three rings and a single double bond.

Preparative GC of Ag-13 monitored by bioassays in combination with active monoene fraction, Ag-9, led to the isolation of a component (Table 3) that was shown to be identical to (*Z,E*)-α-farnesene, the major trail orientation pheromone, by direct comparison with a synthetic sample (Vander Meer et al., 1981). (*Z,E*)-α-Farnesene is present in the Dufour's gland of *S. invicta* at about 6 ng per worker (Vander Meer et al., 1981).

Results of the Y-tube olfactometer tests (Table 4), confirmed those of the point source bioassay in that a combination of (*Z,E*)-α-farnesene and component C-1 was required for attraction. Neither component had significant activity when tested alone. In the specific behavior of attraction, the mixture was indistinguishable from an equivalent amount of Dufour's gland extract. Therefore, the combination of (*Z,E*)-α-farnesene and C-1 (at a ratio of 6 ng:75 pg/WE, respectively) is responsible for the attraction or recruitment of worker ants. However, in the point source bioassay, the two-component mixture elicited only 85% of the activity found with an equivalent amount of Dufour's gland extract. The two natural homofarnesenes did not enhance the activity of C-1 in the olfactometer bioassay (Table 4).

Other components known to be present in Dufour's gland extracts, hep-

TABLE 3. POINT SOURCE RECRUITMENT BIOASSAY RESULTS FOR PREPARATIVE GAS CHROMATOGRAPHY FRACTION Ag-13

Preparative GC fraction[a]	Recruitment ranking[b]
Ag-13A	3.7 ± 1.4
Ag-13B	11.0 ± 3.8
Ag-13C	53.7 ± 5.3
Ag-13D	23.7 ± 11.1
Ag-13E	14.0 ± 6.4
Ag-13F	11.8 ± 3.4
Ag-(9 + 13)	55.3 ± 20.2

[a] All samples and Dufour's gland extracts were tested at 1 WE. All fractions were bioassayed with an equivalent amount of Ag-9.

[b] Mean response (± SE) based on Dufour's gland response = 100 and solvent response = 0 (N = 6).

TABLE 4. OLFACTOMETER RESULTS FOR ORIENTATION AND RECRUITMENT TRAIL PHEROMONE COMPONENTS

Sample tested[a]	Response (%)[b]
Dufour's gland extract	79.5 ± 2.9 A
C-1	60.0 ± 3.0 B
(*Z*,*E*)-α-farnesene	63.0 ± 2.2 B
C-1 + (*Z*,*E*)-α-farnesene	76.5 ± 1.4 A
C-1 + (*Z*,*E*) + (*E*,*E*)-α-homofarnesenes	59.4 ± 1.5B

[a] All samples were tested at 1 WE.
[b] Mean percent response (±SE) to the sample side of the olfactometer ($N = 5$). Results with different letters are significantly different ($P < 0.05$).

tadecane, nonadecane, homofarnesenes, and five heptacosanes, (Vander Meer et al., 1981; Barlin et al., 1976), were inactive in the point source bioassay by themselves and at physiological levels did not enhance the activity of the (*Z*,*E*)-α-farnesene and C-1 mixture.

DISCUSSION

Ant recruitment systems use a wide variety of mechanisms ranging from the tandem running first reported for *Leptothorax acervorum* (Möglich et al., 1974) to the multiple recruitment systems of the African weaver ant *Oecophylla longinoda* (Hölldobler and Wilson, 1978). Tandem running is initiated when the recruiter or calling ant releases an attractive secretion from its poison sac through the sting. When the responding worker touches the calling ant, tandem running occurs. Only a single nestmate is recruited, and it is suggested that tandem running is the evolutionary precursor to odor trail communication. Tandem running has since been observed in ponerine (Hölldobler and Traniello, 1980) and formicine (Hölldobler, 1974) ant species. At the other end of the spectrum is the highly evolved and complex weaver ant system, which involves multiple glandular secretions combined with a variety of tactile signals to cope with a large number of context related recruitment situations (Hölldobler and Wilson, 1978). Examples of recruitment mechanisms that fit in between the two extremes mentioned above have been reviewed by Hölldobler (1978). In examples where more than one gland is involved in recruitment, it is likely that more than one chemical or blend of chemicals is required; i.e. *Myrmica rubra* lays an odor trail with its poison sac contents and deposits attractive spots with the contents of its Dufour's gland (Cammaerts-Tricot, 1974).

S. invicta has a highly evolved and efficient mass recruitment system with

the Dufour's gland as the sole source of the chemicals responsible for releasing this behavior (Wilson, 1959, 1962a,b). *S. invicta* is, to our knowledge, the first example reported in which different chemicals produced in the same exocrine gland control subcategories of trailing behavior. The mass recruitment behaviors elicited by the Dufour's gland contents include attraction, orientation (Wilson, 1962c), and orientation priming (Vander Meer et al., submitted). In addition, depending on the context, Dufour's gland secretions can elicit colony emigration, alarm recruitment, and settling (Wilson, 1962c).

While it was clear that Dufour's gland secretions had multiple functions, it was not evident until this report that different chemical combinations originating from the same gland released different behaviors. This was because previous research on the trail pheromone of *S. invicta* did not differentiate between subcategories. The bioassay usually consisted of streaking test material on a piece of paper and then substituting it for a portion of an already established trail to a food source (Barlin et al., 1976; Jouvenaz et al., 1978; Vander Meer et al., 1981; Williams et al., 1981). This was a good bioassay that gave unambiguous results; however, only the orientation element of the total trail pheromone response was measured. This became clear when the activity of the orientation components was compared to Dufour's gland extracts in two bioassays designed to quantify the recruitment subcategory of the trail pheromone. These initial observations demonstrated that the chemicals isolated and identified as having orientation activity did not, by themselves, elicit recruitment.

In addition to attraction, the point source bioassay measured the settling or aggregation (Wilson, 1962c) behavior of the workers to Dufour's gland extracts or test samples. This bioassay could effectively test up to 10 samples at one time and was used to guide the chemical separation and isolation of the bioactive components. Initially, a combination of three classes of hydrocarbons (saturated, monoenes, and di- to tetraenes) was found to give the best bioassay results; however, quantitative analysis of the saturated hydrocarbon fraction showed that there were exceptionally large amounts of the species-specific saturated hydrocarbons (53 μg/WE) ubiquitous to *S. invicta* (Vander Meer et al., 1982). Previously Thompson et al. (1981) showed that three to four times less material (10–20 μg) released aggregation and biting behaviors in *S. invicta* workers. However, quantitative analysis of these same hydrocarbons from Dufour's gland extracts showed that the hydrocarbons were present at only 16–20 ng per worker. Bioassays of the active unsaturated classes of hydrocarbons, along with the saturated hydrocarbons at the low concentrations found in the Dufour's gland, did not enhance the activity of the unsaturated fractions. Consequently, we felt justified in removing the saturated hydrocarbons from consideration as components of the recruitment part of the trail pheromone.

Further point source bioassays and chemical separations led to: (1) the isolation of a single homosesquiterpene, C-1, with three rings and a single dou-

ble bond, that was present in minute amounts (ca. 75 pg/Dufour's gland WE) and (2) the previously isolated orientation pheromone, (*Z*,*E*)-α-farnesene (ca. 6 ng/Dufour's gland WE). The combination of the two isolated chemicals at physiological concentrations gave 85% of the recruitment activity of an equivalent amount of Dufour's gland extract. These results confirmed that different behaviors associated with the trail pheromone are elicited by different combinations of chemicals derived from the Dufour's gland.

The recruitment point source bioassay was very useful during the isolation and purification of the recruitment pheromone complex, due to its simplicity and reproducibility. However, this bioassay detected multiple behaviors (e.g., attraction, aggregation, and mastication). This problem was avoided by using a Y-tube olfactometer bioassay (Figure 1), which measured only worker attraction. The response of the ants to a mixture of C-1 and (*Z*,*E*)-α-farnesene was indistinguishable from the response to an equivalent amount of Dufour's gland extract (based on the amount of (*Z*,*E*)-α-farnesene). Therefore, this mixture is responsible for the attractive properties of the Dufour's gland contents. Interestingly, the two homofarnesenes did not enhance the attractive qualities of C-1, which indicates an as yet unknown function for these compounds.

The chemistry of ant trail pheromone systems has been the subject of research for many years (Attygalle and Morgan, 1985). Several leaf-cutting ants use methyl 4-methylpyrrole-2-carboxylate produced in their poison gland (Riley et al., 1974). In another *Atta* and several *Myrmica* species, 3-ethyl-2,5-dimethylpyrazine (Cross et al., 1979; Evershed et al., 1982), is the trail following substance. Another pyrazine, 2,5-dimethylpyrazine is a trail following pheromone of *Tetramorium caespitum* (Attygalle and Morgan, 1983). The true trail pheromone of the pharoah's ant was identified as (+)-(3*S*,4*R*)-3,4,7,11-tetramethyltrideca-(6*E*,10*Z*)-dienal (faranal; Ritter et al., 1977). Another aldehyde, (*Z*)-9-hexadecenal is part of the trail pheromone complex of *Iridomyrmex humilus* (Van Vorhis Key and Baker, 1982). Several fatty acids found in the rectal fluid of *Lasius fuliginosus* (Huwyler et al., 1975) and *Pristomyrmex pungens* (Hayashi and Komae, 1977) have been demonstrated to be trail pheromones.

In all of the known examples cited above, a trail-following (i.e., orientation) bioassay was used to measure the ants' response to isolated chemicals. The results presented in this paper demonstrate the pitfalls inherent when a single bioassay is used to measure a complex behavioral event (i.e., trailing or mass recruitment) and that care must be taken to devise bioassays that clearly define the behavior of interest. We have shown for the first time that different exocrine products release different behaviors associated with mass recruitment and that subcategories of trailing behavior can be controlled by different blends of chemicals produced in the same exocrine gland.

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REFERENCES

ALVAREZ, F.M., VANDER MEER, R.K., and LOFGREN, C.S. 1987. Synthesis of homofarnesenes: Trail pheromone components of the fire ant, *Solenopsis invicta. Tetrahedron.* 43:2897–2900.

ATTYGALLE, A.B., and MORGAN, E.D. 1983. Trail pheromone of the ant *Tetramorium caespitum L. Naturwissenschaften* 70:364–365.

ATTYGALLE, A.B., and MORGAN, E.D. 1985. Ant trail pheromones. *Adv. Insect Physiol.* 18:1–30.

BANKS, W.A., LOFGREN, C.S., JOUVENAZ, D.P., STRINGER, C.E., BISHOP, P.M., WILLIAMS, D.F., WOJCIK, D.P., and GLANCEY, B.M. 1981. Techniques for collecting, rearing, and handling imported fire ants. U.S. Dept. Agric., Sci. and Ed. Admin., Advances Agric. Tech., AAT-S-21, 9 pp.

BARLIN, M.R., BLUM, M.S., and BRAND, J.M. 1976. Fire ant trail pheromones: Analysis of species specificity after gas chromatographic fractionation. *J. Insect Physiol.* 22:839–844.

CAMMAERTS-TRICOT, M.C. 1974. Piste et phéromone attractive chez la fourmi *Myrmica rubra. J. Comp. Physiol.* 88:373–382.

CHRISTIE, W.W. 1973. Lipid Analysis. Pergamon Press, Oxford. 338 pp.

CROSS, J.H., BYLER, R.C., RAVID, U., SILVERSTEIN, R.M., ROBINSON, S.W., BAKER, P.M., DE OLIVEIRA, J.S., JUTSUM, A.R., and CHERRETT, J.M. 1979. The major component of the trail pheromone of the leaf-cutting ant, *Atta sexdens rubropilosa* Forel. *J. Chem. Ecol.* 5:187–203.

EVERSHED, R.P., MORGAN, E.D., and CAMMAERTS, M.C. 1982. 3-Ethyl-2,5-dimethylpyrazine, the trail pheromone from the venom gland of eight species of *Myrmica* ants. *Insect Biochem.* 12:383–391.

HAYASHI, N., and KOMAE, H. 1977. The trail and alarm pheromones of the ant, *Pristomyrmex pungens* Mayr. *Experientia* 33:424–425.

HÖLLDOBLER, B. 1974. Communication by tandem running in the ant *Camponotus sericeus. J. Comp. Physiol.* 90:105–127.

HÖLLDOBLER, B. 1978. Ethological aspects of chemical communication in ants. *Adv. Study Behav.* 8:75–115.

HÖLLDOBLER, B., and TRANIELLO, J. 1980. Tandem running pheromone in ponerine ants. *Naturwissenschaften* 67:360.

HÖLLDOBLER, B., and WILSON, E.O. 1978. The multiple recruitment systems of the African weaver ant *Oecophylla longinoda* (Latreille) (Hymenoptera: Formicidae). *Behav. Ecol. Sociobiol.* 3:19–60.

HUWYLER, S., GROB, K., and VISCONTINI, M. 1975. The trail pheromone of the ant *Lasius fuliginosus*: Identification of six components. *J. Insect Physiol.* 21:299–304.

JOUVENAZ, D.P., LOFGREN, C.S., CARLSON, D.A., and BANKS, W.A. 1978. Specificity of the trail pheromones of four species of fire ants, *Solenopsis* spp. *Fla. Entomol.* 61:244.

LOK, J.G., CUPP, E.W., and BLOMQUIST, G.J. 1975. Cuticular lipids of the imported fire ants, *Solenopsis invicta* and *richteri. Insect Biochem.* 5:821–829.

MÖGLICH, M., MASCHWITZ, U., and HÖLLDOBLER, B. 1974. Tandem calling: A new kind of signal in ant communication. *Science* 186:1046–1074.

NELSON, D.R., FATLAND, C.L., HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1980. Reanalysis of the cuticular methylalkanes of *Solenopsis invicta* and *Solenopsis richteri. Insect Biochem.* 10:409–418.

RILEY, R.G., SILVERSTEIN, R.M., CARROLL, B., and CARROLL, R. 1974. Methyl 4-methylpyrrole-2-carboxylate, a volatile trail pheromone from the leaf cutting ant *Atta cephalotes. J. Insect Physiol.* 20:651–654.

RITTER, F.J., BRUGGEMANN-ROTGANS, I.E.M., VERWIEL, P.E.J., PERSOONS, C.J., and TALMAN, E. 1977. Trail pheromone of the Pharaoh's ant *Monomorium pharaonis*; isolation and identification of faranal, a terpenoid related to juvenile hormone II. *Tetrahedron Lett.* 2617–2618.

THOMPSON, M.J., GLANCEY, B.M., ROBBINS, W.E., LOFGREN, C.S., DUTKY, S.R., KOCHANSKY,

J., VANDER MEER, R.K., and GLOVER, A.R. 1981. Major hydrocarbons of the postpharyngeal glands of mated queens of the red imported fire ant *Solenopsis invicta. Lipids* 16:485–495.

VANDER MEER, R.K., GHATAK, U.R., ALAM, S.K., and CHAKRABORTI, P.C. 1979. (±)-Des-*N*-morphinan: A unique bridged hydrocarbon attractant for the rhinoceros beetle, *Oryctes rhinoceros*; and development of an olfactometer. *Environ. Entomol.* 8:6–10.

VANDER MEER, R.K., WILLIAMS, F.D., and LOFGREN, C.S. 1981. Hydrocarbon components of the trail pheromone of the red imported fire ant, *Solenopsis invicta. Tetrahedron Lett.* 22:1651–1654.

VANDER MEER, R.K., GLANCEY, B.M., and LOFGREN, C.S. 1982. Biochemical changes in the crop oesophagus and postpharyngeal gland of colony-founding red imported fire ant queens (*Solenopsis invicta*). *Insect Biochem.* 12:123–127.

VAN VORHIS KEY, S.E., and BAKER, T.C. 1982. Specificity of laboratory trail-following by Argentine ant, *Iridomyrmex humilis* (Mayr) to (*Z*)-9-hexadecenal, analogs and gaster extracts. *J. Chem. Ecol.* 8:1057–1063.

WALSH, C.T., LAW, J.H., and WILSON, E.O. 1965. Purification of the fire ant trail substance. *Nature* 207:320–321.

WILLIAMS, F. 1985. Hydrocarbon components of the trail pheromone of the red imported fire ant (the search for a more environmentally safe control method). Master's thesis. University of Florida, Gainesville, Florida. April 1985.

WILLIAMS, H.J., STRAND, M.R., and VINSON, S.B. 1981. Trail pheromone of the red imported fire ant *Solenopsis invicta* (Buren). *Experientia* 37:1159–1160.

WILSON, E.O. 1959. Source and possible nature of the odour trail of fire ants. *Science* 129:643–644.

WILSON, E.O. 1962a. Chemical communication among workers of the fire ant *Solenopsis saevissima* (Fr. Smith). 1. The organization of mass foraging. *Anim. Behav.* 10:134–147.

WILSON, E.O. 1962b. Chemical communication among workers of the fire ant *Solenopsis saevissima* (Fr. Smith). 2. An information analysis of the odour trail. *Anim. Behav.* 10:148–158.

WILSON, E.O. 1962c. Chemical communication among workers of the fire ant *Solenopsis saevissima* (Fr. Smith). 3. The experimental induction of social responses. *Anim. Behav.* 10:159–164.

WILSON, E.O. 1971. The Insect Societies. Belknap Press of Harvard University, Cambridge, Massachusetts. 548 pp.