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Behavioural and chemical investigation of trail pheromone from the termite *Reticulitermes hesperus* Banks (Isopt., Rhinotermitidae)

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Abstract: A single active fraction, eliciting both induction of trail-following and trail orientation, was isolated by HPLC from dichloromethane extracts of excised fourth and fifth sternites of *Reticulitermes hesperus* workers. GC separation of the active high performance liquid chromatography (HPLC) fraction and subsequent bioassays indicated that a single major component is responsible both for induction of trail-following and for orientation of termites on the trail. Solvent extracts of intact termite bodies and excised sternites elicited greater trail-following activity than extracts of excised abdomens or thoraxes not separated from heads. Workers from two geographically separate colonies responded similarily to dichloromethane extracts of intact bodies and excised sternites from members of either colony, providing no evidence of extractable colony-recognition compounds, although the results suggest that different colonies may produce different quantities of trail pheromone.

1 Introduction

Trail-following in termites and ants involves a series of complex behavioural responses. These include recruitment to the trail (TRANIELLO, 1982), induction of orientation (VANDER MEER et al., 1988), lateral orientation within the trail space (BOSSERT and WILSON, 1963) and longitudinal orientation along the trail (GRACE et al., 1988). These subcatagories can be further defined in terms of chemokinesis and chemotaxis (GRACE et al., 1988; KENNEDY, 1986). Studies by RUNCIE (1987) with *Reticulitermes flavipes* (Kollar), and by TRANIELLO (1982) and TRANIELLO and BUSHER (1985) with *Nasutitermes coastalis* (Holmgren) indicate that different compounds in a chemical trail may be responsible for eliciting trail-recruitment and orientation responses. In the fire ant *Solenopsis invicta* Buren, different subcatagories of trail-following are released by different compounds found in the Dufour's gland (VANDER MEER et al., 1988). However, these behaviours can also be mediated quantitatively, at least in laboratory assays, by the concentration of crude glandular extract (GRACE et al., 1988) or of a single active component (HALL and TRANIELLO, 1985).

Reticulitermes spp., secrete trail pheromone from the sternal gland, located in the anterior portion of the fifth abdominal segment, beneath the fourth sternite (QUENNEDAY, 1971; SMYTHE and COPPEL, 1966). Solvent extracts of the fourth and fifth sternites readily elicit trail orientation (GRACE et al., 1988; HOWARD et al., 1976). The active trail compound (Z,Z,E)3,6,8-dodecatrien-1-ol was identified by MATSUMURA et al. (1968) from *R. virginicus* Banks, and by YAMAOKA et al. (1987) and TOKORO et al. (1991) from *R. speratus* Kolbe. The same or a similar unsaturated alcohol was reported in *R. santonensis* (Feytaud) by RITTER and COENEN-SARABER (1969), and was identified by TOKORO et al. (1989, 1992a) as the trail pheromone of *Coptotermes formosanus* Shiraki. KAIB et al. (1982), MOORE (1974) and PRESTWICH et al. (1984) have suggested that species specificity may be conferred by the presence of additional minor components in termite trails. It is also possible that geographically separated species may not have evolved pheromone specificity.

A weakness of pheromone identifications from *Reticulitermes* spp. is that compounds have been isolated by homogenizing large numbers of intact termites, including the gastro-intestinal tract. Dodecatrienols and other compounds eliciting trail-following have also been isolated from both sound and decayed wood (ESENTHER et al., 1961; HONDA et al., 1975; GRACE and WILCOX, 1988; MATSUMURA et al., 1968; RITTER and COENEN-SARABER, 1969) and might be present in termite gut contents. HOWARD et al. (1976) addressed this problem by comparing the trail-following activity of solvent extracts of intact termites, sternites 4–6, tergites 4–6, heads, and legs. Only extracts of the sternites and of intact termites proved active in behavioral assays, and their activity was equivalent. TOKORO et al. (1992a, 1992b) took a similar approach in identifying the trail pheromone of *C. formosanus* from whole-body solvent extracts, and demonstrated that the biological activity of such extracts was equivalent to that of extracts of the excised fifth sternite.

The present study was initiated to isolate and characterize the trail pheromone of the western subterranean termite, *R. hesperus* Banks, from excised sternites. An earlier report (GRACE et al., 1988) examined the concentration-mediated orientation responses of *R. hesperus* to crude extracts containing trail pheromone. We discuss here the results of a series of behavioural studies conducted to determine (i) whether extraction of excised

sternites and high performance liquid chromatography (HPLC) separation are effective methods of collecting and purifying trail pheromone from *R. hesperus*, (ii) whether bioassay responses of workers from different colony sources are comparable, and (iii) whether separate components of the sternal gland secretion are responsible for induction of trail-following and orientation.

2 Materials and methods

2.1 Source of insects

R. hesperus colonies were collected from four locations in Alameda County, California, USA. The first colony (Berkeley) was removed from a Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, board along a residential driveway in the city of Berkeley; the second (Oakland) from Douglas-fir floor joists in a residence in the city of Oakland; the third (Richmond) from a redwood, *Sequoia sempervirens* (D. Don) Endl., planter box behind a residence in the city of Richmond; and the fourth (Gilman) from Douglas-fir wall framing in a residence in Berkeley.

Termites were removed from the wood and kept in colony groups in plastic trays in a humidity chamber (21–25°C, 94±5% RH) (GRACE, 1986a). Only workers, externally undifferentiated individuals older than the third instar as determined by size, were dissected or used in behavioral assays. With the exception of the experiment on inter-colony differences in behavior, individuals from a single colony were used throughout each extraction, sequential fractionation, and series of bioassays.

2.2 Pheromone extraction and bioassay

Sternal gland extracts were prepared by excising the fourth and fifth sternites from 10 to 700 *R. hesperus* workers immobilized by exposure to dry ice, and soaking these sternites in dichloromethane. Extracts of other body tissues were prepared similarily.

Bioassays were performed on tracing papers laid on a glass surface, uniformly illuminated by overhead fluorescent lighting (13.5–19.5 foot-candles), at a room temperature of 21–25°C. In each assay, a straight artificial trail 200 mm length (1–2 mm in width) was drawn on Monroe No. 41 tracing paper with a syringe containing 1 μl of solution per 50 mm of trail. The solvent was allowed to evaporate for *c.* 15 s, and a single *R. hesperus* worker was gently deposited from a small glass vial onto one end of the trail. Each insect and each trail were used only once to prevent any effects from trail reinforcement or behavioral conditioning.

Two types of bioassays were performed — combined induction plus orientation, and orientation alone. In the combined induction and orientation assay, the forward distance travelled by a termite worker in 30 s on a 200 mm experimental trail was recorded. Twenty-five individual assays were performed with each extract, HPLC fraction and recombined HPLC fractions.

To test for orientation effects following induction of trail-following, we measured the response to the experimental fraction of an insect already engaged in trail-following. A straight 100 mm trail of the test HPLC fraction (total of 2 μl) was preceded by an equivalent 100 mm trail of the fraction found to be most active in the induction plus orientation assay. A worker was deposited on the active portion of the trail (first 100 mm), and the distance continued onto the test portion (second 100 mm) without deviating from the trail or reversing direction was recorded. Ten individual orientation assays were performed with each HPLC fraction, and with recombined HPLC fractions.

In each experiment, a separate series of assays was performed with dichloromethane control trails. Mean distances travelled on the artificial trails were compared by analysis of variance (ANOVA) and means significantly different at the 0.05 level separated by the Ryan–Einot–Gabriel–Welsch (REGW) multiple F test (SAS INSTITUTE, 1982).

2.3 Morphological source of pheromone

An experiment was performed to confirm that extraction of the fourth and fifth sternites was an efficient method of collecting trail pheromone. Ten intact *R. hesperus* worker bodies, abdomens, thoraxes-plus-heads, and excised fourth and fifth sternites were soaked in 1 ml dichloromethane for 1 min. These solutions (1×) and their ten-fold dilutions (0.1×) were assayed as described above for induction of trail-following plus orientation.

2.4 Inter-colony behavioral differences

This experiment was performed to test whether workers from two *R. hesperus* colonies responded differently to solvent extracts of individuals from their own and a second geographically separate colony. Ten whole bodies and ten excised fourth and fifth sternites from the Oakland and Richmond colonies were soaked in 1 ml dichloromethane (per group of ten) for 24 h. Two dilutions of each solution were assayed as described above for induction and orientation activity. Mean responses of 25 workers from each colony to extracts of their own and other colony members were compared at the 0.05 level by *t*-test (SAS INSTITUTE, 1982).

2.5 HPLC-GC separation

In preliminary separations, fractions from dichloromethane extracts of up to 300 excised *R. hesperus* fourth and fifth sternites were separated on an SiO_2 column (mobile phase: pet.ether : ether, 5 : 2), and assayed for induction and orientation activity. In each case, only a single fraction or two adjacent fractions proved active (GRACE, 1986b). Active fractions were further purified by SiO_2 TLC monitored by bioassay before injection on a HPLC column (SiO_2 25 cm × 5 mm ID; ambient temperature; mobil phase; CH_2Cl_2; flow rate, 1 ml/min; detection 230 nm UV). However, comparison of bioassay results to those obtained from direct HPLC separation of the crude extract indicated that initial purification steps were unnecessary (GRACE, 1986b), and they were omitted in this study in favor of direct injection on the HPLC column of extracts of 500–700 excised fourth and fifth sternites.

In this study, HPLC fractions from injection of a dichloromethane extract of 600 excised sternites were collected over 5 min intervals. These fractions were assayed for combined induction plus orientation activity (1× and 0.1×), and for orientation activity alone, as described above. Fractions were assayed both individually and in combination to test for synergism or inhibition.

The fraction exhibiting greatest biological activity from an HPLC separation of a dichloromethane extract of 700 sternal glands was further separated on a Shimadzu GC-9A gas chromatograph (SE-30, 2 m × 3 mm ID glass column; column temperature, 180°C; det. and inj. temp, 250°C; flow rate 50 ml/min; N_2 det. FID). Fractions were collected over 5 min intervals by removing the detector, attaching a short length of teflon tubing and collecting in U-shaped glass tubes held in dry ice. Peak retention times were determined by injection on a capillary column in the same chromatograph (SPB-5 fused silica capillary column, 30 m × 0.25 mm ID; 0.25 μm film;

Table 1. Distances travelled in 30 s by R. hesperus *workers on 200 mm artificial trails drawn with two concentrations of solvent extracts of body tissues*[a]

Tissue	Mean distance ± SEM (mm)[b] 1×	0.1×
Intact body	185.80 ± 6.96a	138.28 ± 12.80a
Sternites 4+5	189.32 ± 7.42a	130.80 ± 14.10a
Abdomen	167.28 ± 11.06ab	79.24 ± 12.08b
Thorax-plus-head	141.76 ± 12.30b	1.68 ± 0.65c
Control (CH_2Cl_2)	–	2.00 ± 0.75c

[a] 1×, tissues from 10 workers extracted in 1 ml dichloromethane for 1 minute, applied at the rate of 1 µl per 50 mm of trail.
[b] $N = 25$; SEM, standard error of the mean; means in the same column followed by different letters are significantly different at the 0.05 level (ANOVA, REGW multiple F test).

column temperature, 60–200°C; 2.5°C/min; det and inj. temperature 250°C; flow rate, 50 ml/min; N_2 det. FID).

3 Results and discussion

Comparison of the biological activity of different termite body tissues (table 1) indicates that dichloromethane extraction of excised fourth and fifth sternites is an effective method of collecting trail pheromone. Although undiluted (1×) extracts of all tissues elicited induction plus orientation, the activity elicited by the excised sternites and the intact body extract was significantly different from, and greater than, that of the thorax-plus-head extract. When 10-fold dilutions (0.1×) were assayed, intact bodies and excised sternites were not different in activity, but both differed significantly from the less-active abdomen and thorax-plus-head extracts. At this 10-fold dilution, the activity of the thorax-plus-head extract was not different from the dichloromethane control.

The reduced activity of abdominal extracts in comparison to intact bodies and excised sternites (table 1) was unexpected. It may be that compounds extracted from internal tissues, hemolymph, or gut contents (all exposed by excision of the abdomen) inhibited the trail-following response. Extraction of either excised sternites or intact bodies may remove fewer extraneous compounds.

Dichloromethane extracts of intact termite bodies and of excised fourth and fifth sternites elicited similar behavioral responses in workers from two different *R. hesperus* colonies (table 2). The only significant differences in response to extracts of colonies from different geographic locations occurred with the 10-fold dilutions (0.1×) of the whole-body extracts. Termite workers from both colonies walked farther on trails drawn with extracts of intact Oakland workers than on the corresponding Richmond trails. This suggests that quantitative differences in pheromone production may exist among *R. hesperus* colonies, although these differences did not occur with extracts of the fourth and fifth sternites. Behavioral differences have been documented among termite colonies (CARTER et al., 1972; SU and LA FAGE, 1984), possibly due to differences in colony cast composition and cycles of molting from one instar to the next (ESENTHER, 1977). Thus, pheromone production might vary between colonies or within a single colony over time.

When HPLC fractions from sternal gland extracts (table 3) were recombined and assayed for induction of trail-following plus orientation, activity above that of the solvent control was detected only when the individually active fractions were included in the combination. Combinations of inactive fractions did not induce trail-following, nor did they elicit orientation among workers already following trails (table 3).

In the orientation assays (table 3), only the HPLC fraction (fraction 7) also eliciting induction was significantly different from the solvent control. Thus, at the level of separation achieved with HPLC, there is no synergism among compounds present in inactive fractions. Either a single compound or compounds eluting in the same period (30–35 min) elicit both induction of trail-following and orientation.

Preparative gas chromatography was performed with the active HPLC fraction from an extract of 700 excised sternites. Five GC fractions were collected in sequence, each over five minutes. The yield was insufficient to perform a full series of trail-following assays, and GC fractions were assayed in a qualitative manner by drawing two or three 100 mm lines on tracing paper with several different dilutions of each fraction and observing the behavior of five to ten termite workers deposited individually at one end of a trail. Any movement greater than a single body length along the trail axis was taken to indicate activity. The two GC fractions collected between 15–20 and 20–25 min after injection were both highly active. Capillary GC indicated a major peak overlapping these two fractions (GRACE, 1986b). Comparative injection of a synthetic compound, (E,Z and E,E)6,8-dodecadien-1-ol, which elicits trail-following in *R. hesperus* (J. K. GRACE and M. KIM, unpubl.), resulted in two associated peaks with similar, although not identical, retention times to this single peak (GRACE, 1986b). The structure of this major component has not yet been elucidated.

Although we have demonstrated the feasibility of isolating trail pheromone from excised sternites by sequential fractionation, the small amounts of material available precluded identification of the pheromone components. This problem also exists with attempts to isolate pheromone from naturally laid trails (TSCHINKEL and CLOSE, 1973). Yet, isolations and identifications from homogenized whole organisms leave open the possibility of contamination by non-pheromonal termite attractants and arrestants. A comparative approach employing isolations from all three sources (MCDOWELL and OLOO, 1984) may be the best solution to this problem. Biological activity of the isolated chemical fractions should be monitored by rigorous behavioral assays, not only for a generalized trail-following response, but also for the specific subcatagories of behavior (GRACE, 1991; GRACE et al., 1988; HALL and TRANIELLO, 1985; VANDER MEER et al., 1988) contributing to the overall response.

Table 2. *Distances travelled in 30 s by* R. hesperus *workers from two colonies on 200 mm artificial trails drawn with solvent extracts of body tissues from each colony*

Colony responding	Concentration[a]	Tissue	Mean distance ± SEM (mm)[b] Oakland source	Richmond source
Oakland	1×	Intact body	191.36 ± 4.77	176.04 ± 8.62
		Sternites 4+5	197.98 ± 2.20	181.40 ± 8.58
	0.1×	Intact body[c]	140.52 ± 14.05	76.36 ± 12.61
		Sternities 4+5	143.80 ± 14.08	152.24 ± 11.41
Richmond	1×	Intact body	182.80 ± 6.13	161.48 ± 12.53
		Sternites 4+5	157.16 ± 13.99	188.24 ± 6.71
	0.1×	Intact body[c]	107.12 ± 12.16	61.48 ± 12.43
		Sternites 4+5	150.28 ± 11.75	162.08 ± 11.20

[a]1× concentration, tissues from 10 workers extracted in 1 mL dichloromethane for 24 hours, applied at the rate of 1 μL per 50 mm of trial.
[b]$N = 25$; SEM, standard error of the mean.
[c]mean distances travelled in response to extracts of intact bodies from different colonies are significantly different at the 0.05 level (*t*-test).

Table 3. *Distances travelled on artificial trails by* R. hesperus *workers in two types of bioassays with fractions from HPLC separation of a dichloromethane extract of 600 sternal glands*

HPLC fraction[a]	Mean distance ± SEM (mm)[b] Induction plus orientation assay 1×[c]	Induction plus orientation assay 10×[d]	Orientation assay 1×[e]
1	0.36 ± 0.25c	0 ± 0b	0 ± 0b
2	0.44 ± 0.31c	0 ± 0b	0 ± 0b
3	0.44 ± 0.31c	1.17 ± 1.17b	0 ± 0b
4	0.28 ± 0.28c	0.83 ± 0.83b	0 ± 0b
5	0.64 ± 0.46c	0 ± 0b	1.00 ± 1.00b
6	0.44 ± 0.32c	0 ± 0b	0 ± 0b
7	159.24 ± 13.75a	100.00 ± 0a	89.20 ± 7.42a
8	0.96 ± 0.55c	1.17 ± 1.17b	0 ± 0b
9	0.40 ± 0.28c	0 ± 0b	0 ± 0b
10,11,12	0.88 ± 0.44c	1.17 ± 1.17b	0 ± 0b
All	137.08 ± 13.78b	97.00 ± 3.00a	—
All except 7	0.48 ± 0.34c	1.17 ± 1.17b	0 ± 0b
Control	0.48 ± 0.35c	0.67 ± 0.67b	0.50 ± 0.50b

[a]Fractions numbered in order of elution (5 min periods), applied at rate of 1 μl per 50 mm of trail.
[b]Means in same column followed by different letters are significantly different at the 0.05 level (ANOVA, REGW multiple F test).
[c]$N = 25$; mean distance travelled in 30 s on 200 mm trail; 1× concentration, 5 ml collected over 5 min.
[d]$N = 6$; mean distance travelled in 30 s on 100 mm trail; 10× concentration created by repeatedly overlaying 1× solution.
[e]$N = 10$; fraction 7 was applied to first 100 mm of trail, and experimental fraction to last 100 mm; distance travelled on the last 100 mm (experimental fraction) only was recorded.

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