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ALARM RESPONSES CAUSED BY NEWLY IDENTIFIED COMPOUNDS DERIVED FROM THE HONEYBEE STING[1,2]

ANITA M. COLLINS[3] and MURRAY S. BLUM[4]

[3]*Bee Breeding and Stock Center Laboratory*
ARS, USDA Baton Rouge, Louisiana 70808
[4]*Department of Entomology, University of Georgia*
Athens, Georgia 30602

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Abstract—Twelve compounds identified from honeybee, *Apis mellifera* L., sting extracts were evaluated in a standardized laboratory test for their effectiveness in eliciting an alarm response from caged worker honeybees. Two—1-decanol and phenol—were judged ineffective as alarm pheromones. The other ten—1-butanol, isopentyl acetate, isopentyl alcohol, 1-hexanol, 2-heptyl acetate, 2-heptanol, 1-octanol, 1-acetoxy-2-octene, 2-nonyl acetate, and 1-acetoxy-2-nonene—produced alarm responses of similar speed and intensity. Three non-sting-derived compounds—β-ionone, methyl benzoate, and *trans*-cinnamaldehyde—caused weak or no responses, indicating that the responses were not simply a reaction to concentrated odoriferous substances.

Key Words—Honeybee, *Apis mellifera*, alarm pheromone, acetate, alcohol, Hymenoptera, Apidae, sting.

INTRODUCTION

In 1978, Blum et al. analyzed extracts of honeybee, *Apis mellifera* L., stings and identified eight previously unreported compounds associated with this structure. The only other sting-derived compound identified prior to that time was isopentyl acetate (IPA) (Boch et al., 1962). These eight compounds,

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n-butyl acetate (nBA), isopentyl alcohol (IPA1), *n*-hexyl acetate (nHA), *n*-octyl acetate (nOA), 2-nonanol (2NL), *n*-decyl acetate (nDA), benzyl acetate (BZA), and benzyl alcohol (BZA1), as well as IPA and 2-heptanone (2HPT), a compound derived from the mandibular glands (Shearer and Boch, 1965), were bioassayed for their effectiveness as alarm pheromones by Collins and Blum (1982). All but nDA and BZA1 were effective in producing alarm response in caged honeybee workers.

Continuing analyses of extracts of honeybee stings have resulted in the identification of 10 more short-chain compounds that can be regarded as potential alarm pheromones (Blum et al., unpublished data). The results of bioassays of the activities of these compounds as alarm pheromones are reported here.

METHODS AND MATERIALS

The method of Collins and Rothenbuhler (1978) was used for bioassays. Caged brood from individual queens (colonies) was emerged in an incubator during a 24-hr period and the young bees placed in glass-fronted wooden cages (described by Kulinčević and Rothenbuhler, 1973) in single-colony groups of 30 workers.

Newly emerged bees are used because they have not yet begun to produce alarm pheromone (Boch and Shearer, 1966) which could interfere with the assay. Although bees of this age are not normally involved in colony defense, in cages they respond with the same, but less intense, behavior as guard bees (Collins, 1980).

During the tests, cages were arranged 15–20 cm apart on shelves in a 35° C walk-in incubator. Tests consisted of separate presentation to the bees of each component diluted in paraffin oil 1 : 9 (v/v). A 0.03-ml sample of this solution was presented under the wire floor of the cage on a small slice of No. 2 cork. The reaction by the bees involved both a flickering of the wings and increased locomotion in the cage. All tests were performed by one observer under double-blind conditions; cage numbers were hidden until after testing, and the cages were rearranged randomly within a set after each complete sequence of tests. A set of eight cages was exposed only to one chemical during the experiment.

The characters were measured as follows: (1) initial activity level—the number of bees moving on the floor, sides, and top of the cage prior to presentation of the stimulus; (2) time to react—the time, in seconds, until a group reaction was seen including flickering of the wings and increased locomotion; (3) initial intensity of this reaction—graded as a weak, medium, strong, or very strong response based on the number and vigor of responding bees; and (4) number of bees engaged in fanning behavior at the end of the test.

Occasionally this fanning included exposure of the Nasonov gland. Following testing, a fifth character was calculated—frequency of no reaction—the number of times in which there was no reaction to the test material. Analysis of the time to react was done on square-root transformed data by least-squares analysis of covariance, adjusted for initial activity level, and by a least significant difference test (LSD). The adjustment of time to react was necessary because bees that are already active tend to respond more quickly and strongly. Spearman's rank correlation coefficients were calculated for all pairs of measures of response (Hollander and Wolfe, 1973). Intensity of the response was analyzed by chi-square and fanning by the t test of equality of two means.

The sting-derived compounds tested included 2-heptanol (2HPA1), 1-octanol (OA1), 1-butanol (BA1), and phenol purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; and 1-acetoxy-2-octene (1AO), 1-acetoxy-2-nonene (1AN), 2-heptyl acetate (2HPA), and 2-nonyl acetate (2NA) which were synthesized and purified by preparative gas chromatography. The assays were done during two 6-day periods, with half of the compounds tested each period, due to space limitation. Bees from two colonies were used for testing. 2-Nonyl acetate was tested during both periods as a control. IPA, also from Aldrich Chemical Co., was used as a control so that comparisons could be made with the group of compounds previously tested (Collins and Blum, 1982).

In addition, three aromatic compounds not produced by worker honeybees, methyl benzoate (MB), *trans*-cinnamaldehyde (tCNM), and β-ionone (βI) (Aldrich Chemical Co.) were included in the study. This was done in order to distinguish between alarm behavior and simple aversive responses to volatile compounds introduced into test cages.

At a later date, two compounds not found in sting extracts, 1-hexanol (HA1) and 1-decanol (DA1) (Aldrich Chemical Co.) were assayed to complete the series of acetates and their alcoholic moieties in the range C_4 (n-butyl) to C_{10} (n-decyl). 2NA was the control and IPA1 (Aldrich Chemical Co.) was the comparison control with this set, rather than IPA. It was necessary to use three colonies to provide sufficient bees, as brood rearing was reduced at this time.

RESULTS

The three test sequences using 2NA were not significantly different in the LSD test, so the data from the three testing periods are presented together. The responses to IPA and IPA1 were not significantly different from those during the 1982 (Collins and Blum) assays, so comparisons were made including all chemicals tested to date.

Table 1 shows the distribution of observations by intensity of response. Two of the sting-derived compounds, DA1 and phenol, and the three foreign compounds, tCNM, MB, and βI, elicited response from the caged bees less than half the time. When a response to these compounds was seen, it was usually a weak one. Responses to the remaining compounds occurred more often, although the intensity levels varied. The compounds were ranked based on the numbers of observations in each category, with a rank of 1 indicating the greatest number of strong or very strong responses and 15 the fewest responses.

The time to react, intensity ranking based on Table 1, and number of bees fanning for each compound tested are shown in Table 2. Mean times to react varied on a continuum from 3.4 to 14.4 sec with only the most extreme values being significantly different from each other. The five chemicals with low levels of response had the slowest mean times to react. The three measures of alarm response (frequency of no response, time to react, and intensity) were significantly correlated at $P < 0.01$ using Spearman's rank correlation (Table 3). Compounds eliciting frequent response got reactions that were faster and

TABLE 1. INTENSITY OF RESPONSE BY CAGED HONEYBEES TO 15 COMPOUNDS TESTED AS ALARM PHEROMONES

Rank[a]	Chemical	No response	Weak	Medium	Strong	Very strong	Total observations[b]
1a	1-hexanol	1	7	31	32	10	81
2b	isopentyl acetate	1	9	41	21	0	72
3b	2-heptanol	0	16	43	13	0	72
4b	isopentyl alcohol	3	19	35	20	4	81
5c	1-acetoxy-2-nonene	0	32	29	11	0	72
6d	1-butanol	6	24	35	7	0	72
7d	1-octanol	6	24	30	3	0	63
8de	2-heptyl acetate	10	26	30	6	0	72
9e	2-nonyl acetate	53	96	65	9	2	144
10f	1-acetoxy-2-octene	14	43	15	0	0	72
11g	phenol	41	24	7	0	0	72
12g	*trans*-cinnamaldehyde	40	28	4	0	0	72
13g	methyl benzoate	46	23	3	0	0	72
14h	1-decanol	57	11	14	7	2	81
15g	β-ionone	61	11	0	0	0	72

[a] Rank was determined by relative number of observations in each category with 1 being the group with the greatest number of strong responses and 15 the group with the greatest number of weak or no responses. Chemicals with the same letter are not significantly different by contingency chi-square.

[b] Eight cages with 30 bees each were tested 3 times a day for 3 days. 2-Nonyl acetate was tested three times to serve as a control for different test dates.

TABLE 2. MEASURES OF RESPONSE OF CAGED HONEYBEE WORKERS TO 12 COMPOUNDS ASSOCIATED WITH STING AND 3 UNRELATED AROMATIC COMPOUNDS[a]

	No. non-reactors total	Least squares $\bar{X}$ time to react (s)[b]	Intensity (rank)[c]	No. of bees fanning[d]
Isopentyl acetate (IPA)	1/72	3.8 a	2	25
2-Heptanol (2HPA1)	0/72	4.4 a	3	141
1-Hexanol (HA1)	1/81	4.5 a	1	319
Isopentyl alcohol (IPA1)	3/81	5.1 ab	4	119
1-Octanol (OA1)	6/63	5.6 ab	7	41
1-Acetoxy-2-octene (1AO)	14/72	5.8 ab	10	127
1-Acetoxy-2-nonene (1AN)	0/72	6.0 ab	5	41
2-Heptyl acetate (2HPA)	10/72	6.2 ab	8	155
1-Butanol (BA1)	6/72	6.5 abc	6	135
2-Nonyl acetate (2NA)	34/144	7.0 bc	9	111
Methyl benzoate[a,e] (MB)	46/72	7.1 bc	13	351
Phenol[e] (P)	41/72	8.3 bcd	11	291
trans-Cinnamaldehyde[a,e] (tCNM)	40/72	9.5 cd	12	138
1-Decanol[e] (DA1)	57/81	10.4 cd	14	22
β-Ionone[a,e] (βI)	61/72	14.4 d	15	259

[a]Methyl benzoate, *trans*-Cinnamaldehyde and β-ionone not associated with sting.
[b]Means followed by the same letter(s) are not significantly different ($P < 0.01$).
[c]Rank taken from Table 1.
[d]Total of all observations from nine tests with each chemical.
[e]These chemicals probably do not function as alarm pheromones based on the frequency of nonreactors.

TABLE 3. SPEARMAN'S RANK CORRELATION COEFFICIENT AMONG 4 MEASURES OF ALARM RESPONSE[a]

	Time to react	Intensity	Fanning
Frequency of no reaction	0.88[b]	0.88[b]	0.07
Time to react		0.94[b]	0.16
Intensity			0.01

[a]Calculations done using data from this paper and Collins and Blum (1982).
[b]Correlation is significant at $P < 0.01$, $df = 10$.

TABLE 4. HONEYBEE COLONY DIFFERENCES[a] IN TIME TO REACT TO VARIOUS ALARM PHEROMONES (VALUES ARE COLONY MEANS IN SECONDS)

Pheromone	Colony A	Colony B
Isopentyl acetate	3.9	4.2
2-Heptanol	3.1	5.6
1-Octanol	3.9	7.3
1-Acetoxy-2-octene	4.7	7.2
1-Acetoxy-2-nonene	5.4	6.7
2-Heptyl acetate	5.4	7.0
1-Butanol	5.6	6.6
2-Nonyl acetate	6.1	8.7

[a]Differences significant at $P < 0.01$ ($F = 37.23$; df 1, 12).

more intense. Fanning level differed by chemical tested, but was not significantly correlated with the other three measures.

In addition, there were significant colony differences. Bees from colony B reacted more slowly to each of the compounds (Table 4) and overall with less sensitivity (Table 5). The heterogeneity chi-square for the intensity was not significant, so the data were pooled. Colony A had a mean of 58 bees seen fanning in response to a compound during the test period, colony B had a mean of 97.3 bees, significantly ($t = 5.18$, $P < 0.01$) more.

DISCUSSION

The evaluation of *trans*-cinnamaldehyde, methyl benzoate, and β-ionone as alarm pheromones was considered to be of critical importance in order to establish unequivocally that the response by the small group of worker honeybees in the test cages was not simply an aversive response to high concentrations of any odoriferous substance. The fact that the bees usually exhibited only weak responses (a category reserved for observed responses that are marginal) or did not respond at all, indicates that the presence of a strongly odoriferous compound is, in itself, insufficient to create an alarm response. There was, however, considerable fanning by the bees in the presence of these three compounds. Both tCNM and βI had been tested by Woodrow et al. (1965), who evaluated a large number of chemicals for attractiveness and repellency to bees for possible application with pesticides. tCNM was moderately repellent, and the bees did fan their wings during exposure.

From the group of sting-derived compounds tested, only the alcohols of

Compound	Colony	Intensity: No response	Weak	Medium	Strong	Very strong	χ^2	*df*
Isopentyl acetate	A	0	2	21	13	0	4.99	3
	B	1	7	20	8	0		
2-Heptanol	A	0	3	26	7	0	8.21*[a]	2
	B	0	13	17	6	0		
1-Octanol	A	1	7	16	3	0	8.86*	3
	B	5	17	14	0	0		
1-Acetoxy-2-octene	A	5	22	9	0	0	1.77	2
	B	9	21	6	0	0		
1-Acetoxy-2-nonene	A	0	10	17	9	0	9.82**	2
	B	0	22	12	2	0		
2-Heptyl acetate	A	3	11	18	4	0	4.08	3
	B	7	15	12	2	0		
1-Butanol	A	1	10	20	5	0	5.33	3
	B	5	14	15	2	0		
2-Nonyl acetate	A	15	24	28	5	0	10.97*	3
	B	19	38	14	1	0		
Methyl benzoate	A	19	15	2	0	0	3.86	2
	B	27	8	1	0	0		
Phenol	A	21	11	4	0	0	0.33	2
	B	20	13	3	0	0		
trans-cinnamaldehyde	A	22	14	0	0	0	4.40	2
	B	18	14	4	0	0		
β-Ionone	A	30	6	0	0	0	0.11	1
	B	31	5	0	0	0		
						summed χ^2	62.73	28
Total	A	117	135	161	46	0		
	B	142	187	118	21	0	26.68**	3
						heterogeneity χ^2	36.05	25

[a] *Significant at $P < 0.05$; ** significant at $P < 0.01$.

C_9 or less, or their acetates, were effective in eliciting an alarm response. The levels of response were not significantly different in speed, but did vary in intensity. All were significantly faster and stronger in their elicited response than were DA1 or phenol. These two compounds are apparently ineffective as alarm pheromones, since they produced no response more than half the time, and the responses that did occur were weak.

The only compound in the acetate–alcohol series of alarm pheromones that did not show an alarm function was DA1. As with its corresponding acetate, which was evaluated with the first group of compounds (Collins and Blum, 1982), in more than half the tests the bees did not respond at all. Including the compounds which had been previously tested, all the acetates and their alcoholic moieties from C_4 (*n*-butyl) to C_9 (*n*-nonyl), plus 1AO, 1AN, and BZA, can be considered as functional alarm pheromones. BZA1 was not effective in eliciting a response. Among the alarm pheromone group, the speeds of the response were not significantly different, but the intensity did differ significantly for some. However, at this juncture no general statement can be made about the relationship of chemical structure and its effect on the intensity of the alarm response.

These assays were based solely on responses with caged young bees using individual chemicals in amounts far exceeding those present in the stings of bees. Guard bees at the hive entrance under more normal conditions might have different response thresholds for the individual compounds. Also, interactions betweeen chemicals presented simultaneously may occur, but were not examined in the present study.

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