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ALARM PHEROMONE PRODUCTION BY TWO HONEYBEE (*Apis mellifera*) TYPES

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Abstract—Of 12 alarm pheromones assayed in European and Africanized honeybees, nine were found in larger quantities in the Africanized population. Isopentyl and 2-heptanone levels were similar in both; 2-methylbutanol-1 was greater in European workers. These differences were not due to age or geographical location. Significant positive correlations between alarm pheromone levels and defensive behavior, especially numbers of stings, were observed.

Key Words—Honeybee, *Apis mellifera*, alarm pheromone, Hymenoptera, Apidae, isopentyl acetate, 2-heptanone, sting, mandibular gland.

INTRODUCTION

A vital part of honeybee colony defense is the communication of alarm through the use of pheromones (Maschwitz, 1964; Collins et al., 1980). Various investigators have identified alarm pheromones by chemical analysis and assay of biological activity. Boch et al. (1962) identified isopentyl acetate (IPA) (previously called isoamyl acetate) as a biologically active alarm pheromone associated with the sting, and Shearer and Boch (1965) reported on the alarm activity of 2-heptanone (2HPT) isolated from mandibular glands in the head. Blum and colleagues (Blum et al., 1978; Blum and Fales, 1988) identified over 80 compounds from the sting apparatus, of which 15 were shown to elicit alarm responses from caged workers (Collins and Blum, 1982, 1983). Pickett et al.

(1982) reported that (Z)-11-eicosen-1-ol was also an important component of the sting pheromone complex.

These initial identifications were made on samples of worker honeybees collected in North America (*Apis mellifera*, European subspecies). Two other subspecies, *A. m. scutellata* (formerly *adansonii*) and *A. m. capensis*, were assayed for IPA and 2HPT by Crewe and Hastings (1976). *A. m. scutellata* exhibits more intense colony defense than the European subspecies. The range of pheromone levels found, however, was within that seen in the European populations and did not prove to be an easily obtained predictor of defensive behavior.

Two other studies (Kerr et al., 1974; Boch and Rothenbuhler, 1974) investigated the relationship of IPA and 2HPT levels with several measures of defensiveness in the same colony of European bees. Levels of IPA did not show a significant correlation with the behavioral assay in either study, even though the more defensive line of Boch and Rothenbuhler had higher levels of IPA assayed. However, Kerr et al. (1974), using Africanized bees, reported that levels of 2HPT were positively correlated with three measures of defensive behavior: time to first sting, time to visible worker response, and number of stings.

The work reported here is from two studies measuring levels of IPA, 2HPT, and the more recently identified components of sting alarm pheromone (Blum et al., 1978; Collins and Blum, 1982, 1983) in two geographical types, European and Africanized bees.[3] These geographical types display quite different levels of defensive behavior (Collins et al., 1982). One study involved sampling from a large number of representative colonies in Louisiana, U.S.A., and Monagas, Venezuela. The second was an experiment controlling for location and age in a smaller number of colonies. Correlations of the pheromone levels with aspects of defensive behavior as measured by a sequenced standardized test (Collins and Kubasek, 1982) were also calculated for the larger study.

METHODS AND MATERIALS

Survey. This experiment used 150 large colonies near Baton Rouge, Louisiana (30°30'N, 91°W), and 147 similar-sized colonies near Maturin, Monagas, Venezuela, (10°N, 63°W). The Louisiana colonies had been established

[3] The European honeybees in this study were from North America. Such bees have in their ancestry representatives of mixed subspecies. Africanized bees are descendants of *A. m. scutellata* bees imported from Africa and their hybrids with various subspecies previously imported into Brazil. Neither the European nor the Africanized bees can correctly be called race, subspecies, stock, or line representatives. We use the term "geographical type" to indicate that the bees we studied showed major characteristics typical of descriptions for temperately or tropically (*A. m. scutellata*) adapted bees.

from various U.S. commercial honeybee stocks. Some of the Venezuelan colonies had originally been headed by European queens but were chosen on the basis that they had not been requeened within at least the past year. This would have been sufficient time for several supersedures with the new queens mating with Africanized drones or for invasion of colonies by Africanized queens. The rest of the Venezuelan colonies had been established from caught swarms.

Ten bees from each colony were collected in alcohol and identified using an updated version of the Daly et al. (1982) morphometric identification procedure. Twenty-five characters were measured and the colonies classified as European, Africanized, or hybrid.

Samples of 30 worker bees of mixed ages per colony were collected from the entrance in a plastic bag and killed by freezing. The sting apparatus and head of each bee were removed from the body by forceps, immersed in 1 ml pesticide-grade methylene chloride with sodium sulfate as a drying agent, and sealed in a crimp-top vial. Three samples of 10 bees each were prepared per colony and stored in a freezer prior to analysis.

One-microliter aliquots of solvent from each vial were injected into a Perkin Elmer Sigma 3 gas chromatograph fitted with two glass columns (6 ft $\times$ $\frac{1}{4}$ in. OD, 2 mm ID, packed with SP1000 on 80–100 mesh Chromasorb W with 10% loading) run as dual compensation. The heat cycle started at 70°C for 3 min and increased 5°C/min up to 120°C. Quantities of butyl acetate, IPA, 2-methylbutanol-1, hexyl acetate, 1-hexanol, 2-heptyl acetate, 2-heptanol, octyl acetate, 1-octanol, 2-nonyl acetate, 2-nonanol, and 2HPT were calculated by electronic integration using a Varian CDS III.

The data were analyzed with an analysis of variance for bee type using the mean value per colony. Colony means were also used for the correlations of pheromone and behavior measures. Data for each chemical were transformed by square root. Differences between means were determined by Tukey's test.

The colonies were also tested using a standardized procedure for measuring colony defense (Collins and Kubasek, 1982). These results were reported in Collins et al. (1982). For the defense test, a picture of the bees around the entrance was taken before testing. Timing of the test began when 0.8 ml of an artificial alarm pheromone (isopentyl acetate in paraffin oil, 1 : 100 by volume) was sprayed over the entrance. The other compounds from the sting had not been identified at the time of development of this test and were not used. At 30 sec, the second photograph was made, and the colony was given a physical jolt by shooting it with a glass marble (19 g, 2.3 cm in diameter) propelled by a slingshot. The third photograph was taken at 60 sec, and the suede targets were moved into place, one 2–6 cm in front of the entrance and a second 25 cm farther away. These two blue suede targets (5 $\times$ 5 cm) were clipped to the arms of a battery-operated device that swung them vertically 20 cm about 120 times per minute during the 60- to 90-sec interval. At 90 sec, the targets were removed

and the final photograph was taken. New targets were used for each test sequence. At a later time, the number of bees in each photograph and the number of stings in the two targets were counted. Each colony was tested twice, on different days, and colony means were calculated.

Controlled Location and Age. Because samples from the extensive survey of the two populations were collected in separate geographic locations (Louisiana and Venezuela), the possibility existed that differences seen were due to environmental differences. Such factors as temperature, food sources, and previous disturbance might influence alarm pheromone levels or components produced. Also, both IPA and 2HPT levels vary with age, as reported by Boch and Shearer (1966, 1967), reaching a peak at ca. two weeks and then decreasing somewhat to a stable level. Whiffler et al. (1988) reported age variation for a number of the other alarm pheromones and associated changes in responsiveness to alarm pheromones.

To control for location and age in these pheromone assays, newly emerged bees from 10 Africanized and 10 European (various sources) colonies from the same apiary in Sarare, Lara, Venezuela (10°N, 69°W) were color-coded for eclosion date by being marked on the thorax with paint and released in the parent colony. Four weeks after emergence, marked bees were collected, held for 24 hr in a small cage in an incubator with sugar syrup and water available, and then killed by freezing. Stings were collected as above, except that only 0.5 ml of solvent was used and an internal standard, methyl octanoate, was added. Three additional sting alarm pheromones were measured, 1-acetoxy-2-octene, 1-acetoxy-2-nonene, and benzyl acetate. Data were analyzed by analysis of variance for bee type using the mean value per colony. Data were transformed by square root.

RESULTS AND DISCUSSION

Survey. All of the colonies in Louisiana were identified as European, but the population in Venezuela included 70 European colonies, 37 Africanized colonies, and six hybrid colonies. This allowed for additional comparisons of differences due to location (Europeans in Louisiana and Venezuela). Three Louisiana colonies and 29 Venezuela colonies were not precisely identified at the 0.90 threshold and were deleted from the data set.

The mean values of micrograms per bee produced for each of the 12 alarm pheromones are shown in Table 1. The sum of all the pheromones measured except 2HPT, which is not part of the sting pheromone complex, and 1-hexanol, for which there were too few observations, is also included. The four classes of bees were not significantly different for levels of IPA, the major sting alarm pheromone component. 2-Heptanone levels are probably not different in

TABLE 1. MEAN MICROGRAMS PER WORKER BEE (± SD) OF 12 ALARM PHEROMONES FOR TWO DISTINCT HONEYBEE TYPES AND THEIR HYBRID[a,b]

	Bee type				
	European		Hybrid,	Africanized,	
Alarm pheromone	Louisiana	Venezuela	Venezuela	Venezuela	F^h
Butyl acetate	0.01 ± 0.03a[i]	0.14 ± 0.22b	0.28 ± 0.16c	0.56 ± 0.35d	117.60**
Isopentyl acetate	1.92 ± 0.85a	1.86 ± 1.00a	2.10 ± 1.13a	2.27 ± 1.35a	1.42 NS
2-Methylbutanol-1	0.47 ± 0.24a	0.43 ± 0.23a	0.43 ± 0.17ab	0.29 ± 0.18b	8.63**
Hexyl acetate	0.04 ± 0.03a	0.18 ± 0.15b	0.33 ± 0.18c	0.61 ± 0.38d	135.12**
1-Hexanol	0.01 ± 0.01a	0.02 ± 0.03b	0.03 ± 0.03b	0.10 ± 0.06c	59.29**
2-Heptyl acetate	0.08 ± 0.05a	0.14 ± 0.08b	0.20 ± 0.07bc	0.23 ± 0.14c	30.97**
2-Heptanol	0.01 ± 0.01a	0.02 ± 0.02b	0.03 ± 0.01c	0.06 ± 0.04c	73.77**
Octyl acetate	0.02 ± 0.03a	0.17 ± 0.20b	0.28 ± 0.24b	0.59 ± 0.48c	91.84**
1-Octanol	0.01 ± 0.02a	0.16 ± 0.50b	0.27 ± 0.12c	0.37 ± 0.36c	61.88**
2-Nonyl acetate	0.03 ± 0.05a	0.04 ± 0.09a	0.06 ± 0.09ab	0.12 ± 0.11b	19.16**
2-Nonanol	0.14 ± 0.18a	0.43 ± 0.71b	0.64 ± 0.27bc	1.93 ± 4.44c	59.09**
2-Heptanone[c]	1.34 ± 0.69a	1.01 ± 0.53b	1.06 ± 0.33ab	1.26 ± 0.55ab	3.79*
Sum	1.46 0.33a	1.77 0.56ab	2.11 0.41bc	2.51 0.64c	4.85**
Number of colonies	64[d]	70[e]	6[f]	37[g]	

[a] Sum is the total quantity of all sting alarm pheromones assayed, except 1-hexanol.
[b] Based on colony means from 3 samples of 10 bees each. Transformed by square root for ANOVA and Tukey's.
[c] This pheromone is produced in the mandibular glands, all others in the Koschewnikow gland associated with the sting.
[d] Isopentyl acetate n = 144; 2-heptanone n = 146.
[e] Hexanol $N = 21$.
[f] Hexanol $N = 3$.
[g] Hexanol $N = 7$.
[h] $**P < 0.01$; $*P < 0.05$; NS $P > 0.05$.
[i] Means within pheromone followed by the same letter are not significantly different.

the European and Africanized types, although the statistical relationships seen here are difficult to decipher. 2-Methylbutanol-1 is produced in greater quantity by European bees, but all the rest of the pheromones and their sum are at higher levels in the Africanized population. The intermediate values of the few hybrids indicate the likelihood of a simple additive genetic control for the production of most of these compounds.

Correlations of the levels of pheromones with the measures of colony defense for all colonies are presented in Table 2. The components of the sting alarm pheromone which occurred at higher levels in the Africanized group were consistently significantly correlated with a more defensive behavior. Levels of

TABLE 2. SIGNIFICANT CORRELATION COEFFICIENTS OF ALARM PHEROMONE LEVELS WITH DEFENSIVE BEHAVIOR IN STANDARD TEST FOR COMBINED POPULATION[a]

Alarm Pheromone	Defensive behavior						
	Seconds to react to		Number of bees on entrance at:				Total stings
	Pheromone	Target	00 sec	30 sec	60 sec	90 sec	
Butyl acetate	−0.499	−0.466	0.148*	0.340	0.374	0.365	0.574
IPA	—	—	—	0.126*	0.142*	0.209	0.125*
Hexyl acetate	−0.521	−0.526	0.217	0.388	0.412	0.366	0.602
1-Hexanol	−0.292	−0.372	—	—	—	—	0.440
2-Heptyl acetate	−0.389	−0.379	0.234	0.321	0.337	0.373	0.463
Heptanol	−0.509	−0.417	0.140*	0.322	0.362	0.351	0.508
Octyl acetate	−0.450	−0.478	0.171*	0.345	0.369	0.313	0.556
Octanol	−0.417	−0.432	—	0.291	0.308	0.283	0.505
2-Nonyl acetate	−0.247	−0.180*	—	0.203	0.223	0.189	0.268
2-Nonanol	−0.452	−0.377	—	0.288	0.317	0.285	0.486
2-Heptanone	—	0.140	—	—	—	—	—
Sum	−0.428	−0.430	0.152*	0.334	0.368	0.378	0.554

[a] Sum is the total quantity of all sting alarm pheromones assayed except 1-hexanol. $P < 0.01$, except * = $P < 0.05$.

2HPT, the mandibular gland pheromone, do not correlate well with behavioral measures. IPA, which was not significantly different between bee types, shows only a low correlation with the number of bees responding to the test stimuli and with the number of stings. The corresponding alcohol, 2-methylbutanol-1, had no significant correlation with the behavior. Levels of 1-hexanol were not correlated with the number of bees responding but were correlated to the speed of the responses and to number of stings. The low correlations to number of bees on the entrance at 00 sec are expected since stimuli for defensive behavior have not been presented at this time.

Controlled Location and Age. From an examination of the data in Table 1, there are differences between the European groups from Louisiana and Venezuela. Nine of the compounds are at higher levels in the Venezuelan group. This difference can be easily explained by the presence of Africanized drifters in the Venezuela European colonies. Morphometric identifications support this, as only 12% of the Louisiana European colonies had one or two bees classed as Africanized, while 42% of the Venezuela European colonies had one to three Africanized individuals.

The levels of alarm pheromones found in worker bees of the two types when geographic location was identical and the age of the collected bees was controlled are shown in Table 3. The results are similar to those found for the initial survey. The major difference is that in the controlled group, IPA occurs in greater quantity in the Europeans. In comparing the actual quantities of IPA found per bee, the bees in the second study have less than half of what was found previously. IPA increases to a peak in bees that are of guarding and beginning-foraging age (ca. 2 weeks) and decreases thereafter (Boch and Shearer, 1966). In the age-controlled collection, the younger bees of guarding age with higher IPA levels were excluded and the 4-week-old bees collected would expectably show decreasing IPA levels.

The two values for 2-heptyl acetate and 2-nonyl acetate are not statistically different in the second study. However, the direction of the difference that is there (Africanized greater than European) is the same as that previously found. Of the three new pheromone components analyzed, 1-acetoxy-2-octene, 1-acetoxy-2-nonene, and benzyl acetate, only the last shows a significant difference with Africanized levels greater than European.

TABLE 3. MEAN MICROGRAMS (± SD) PER WORKER BEE OF 15 ALARM PHEROMONES FROM 4-WEEK-OLD BEES OF BOTH EUROPEAN AND AFRICANIZED TYPE REARED IN SAME APIARY[a]

Alarm pheromone component	Bee type		
	European	Africanized	F^c
Butyl acetate	0.06 ± 0.05	0.66 ± 0.05	73.71**
Isopentyl acetate	1.30 ± 0.09	0.67 ± 0.09	24.90**
2-Methylbutanol-1	0.07 ± 0.02	0.01 ± 0.02	5.05*
Hexyl acetate	0.10 ± 0.05	0.74 ± 0.05	95.15**
1-Hexanol	0.04 ± 0.02	0.17 ± 0.01	55.42**
2-Heptyl acetate	0.09 ± 0.02	0.14 ± 0.02	3.61 NS
2-Heptanol	0.10 ± 0.01	0.16 ± 0.01	12.75**
Octyl acetate	0.16 ± 0.05	0.74 ± 0.06	57.77**
1-Octanol	0.11 ± 0.04	0.49 ± 0.04	42.66**
2-Nonyl acetate	0.11 ± 0.02	0.17 ± 0.02	3.51 NS
2-Nonanol	0.33 ± 0.12	1.40 ± 0.13	38.14**
1-Acetoxy-2-octene	0.31 ± 0.03	0.34 ± 0.03	0.85 NS
1-Acetoxy-2-nonene	0.001 ± 0.001	0.002 ± 0.002	0.10 NS
Enzyl acetate	0.51 ± 0.09	1.16 ± 0.10	23.88***
2-Heptanone[b]	15.46 ± 1.52	14.68 ± 1.59	0.12 NS

[a] Ten colonies of each type were sampled.
[b] See Table 1, footnote [c].
[c] *$P < 0.05$; **$P < 0.01$; NS, not significant.

Therefore, it appears that with the exception of IPA (the major sting alarm pheromone component) and its alcohol, 2-methylbutanol-1, and, possibly, 2HPT (a mandibular gland alarm pheromone), Africanized bees produce greater quantities of nine alarm pheromones. Furthermore, within the species *Apis mellifera*, these higher concentrations of alarm pheromones are associated with more intense levels of colony defense. The correlation of 0.554 (Table 2) between the total amount of alarm pheromone and the total number of stings indicates that 0.31 (r^2) of the variance of numbers of stings is determined in linear fashion with the variance in quantities of alarm pheromone (Snedecor and Cochran, 1967). A higher concentration of pheromone released from the first bee alerted could alert more recruits. Because alarm pheromones mediate the first step in honeybee colony defense (Collins et al., 1980), the influence of a higher concentration of pheromone on the intensity of the defensive response is easily seen and has been documented by Collins et al. (1987b). As the recruited bees also release pheromone through extrusion of the sting and loss of the sting in an intruder, a higher concentration of alarm pheromone would accumulate around Africanized colonies than around equivalent European colonies. This higher level of pheromone would also require a longer time to disperse. Both of these phenomena would account for at least part of the difference in defensive behavior between the two honeybee types. At this time, it does not seem necessary to invoke synergistic effects of differing proportions of the components in the two bee types to account for behavioral differences.

The correlations found between the pheromone produced and the behavior induced suggest that the genes controlling the variation of both of these characters are coadapted, that is, jointly selected for their effects on fitness (Falconer, 1981). It has been further hypothesized that coadapted genes form supergenes by being closely associated on a chromosome (linked) (Dobzhansky, 1970). Such supergene complexes would tend to stay together and could account for the persistence of the intensive African defense phenotype in the Africanized populations.

We have reason to believe that the number of genes involved could be small for each character. Both the behavior (Collins et al., 1984) and the pheromones (Collins et al., 1987a) show genetic correlations. A few genes could determine structure or biochemistry of the central nervous system, resulting in variable behavior. Likewise, very few genes might be needed for the synthesis of the array of very similar compounds that are the alarm pheromones studied.

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