Laboratory Test of the Response to an Alarm Chemical, Isopentyl Acetate, by *Apis mellifera* [1,2]

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ABSTRACT

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A laboratory test was developed to measure the response to isopentyl acetate (IPA) by the honey bee, *Apis mellifera* L. Bees 2–5 days old showed clear responses measured as seconds to react, duration of the reaction, and intensity of the reaction. An interval of 15 min or more between repeated tests was sufficient to produce consistent results. Groups of 10, 25, or 50 bees were adequate to evaluate a reaction, but a group of 5 was not. Increasing concentrations of IPA in paraffin oil (from 1:10,000 to 1:2) produced quicker, longer lasting, and stronger reactions. Measurements of the response by genetically defined bees using the lab test agreed with evaluations of their temperament in the field.

When a beekeeper or some other marauder ventures near a colony of honey bees, *Apis mellifera* L., individuals that are alerted may engage in various sorts of defensive behavior. One such behavior is the release of alarm pheromone which in turn alerts and alarms more bees. Boch et al (1962) identified the major component of the sting alarm pheromone as isopentyl acetate (IPA) and subsequently, Boch and Shearer (1965) identified the mandibular gland alarm pheromone as 2-heptanone (2HPT). These chemicals, when presented to bees in a colony, elicit characteristic behavior patterns associated with alarm and defense.

Investigations such as Stort's (1974) of alarm and defense displayed by whole colonies in the field are complicated by a variety of environmental factors. Such things as weather, foraging conditions, and recent colony history influence the promptness and magnitude of the bees' response to a disturbing stimulus. If measurements of their response could be made in the laboratory using small samples of bees, these variables could be reduced.

Our study was undertaken to design an appropriate laboratory test of response to an alarm chemical—a test which could be used later to examine the genetics of differences in honey bee alarm behavior. For this purpose, we examined how the response would be affected by the number of bees in a test cage, by the length of the interval between repeated tests, by the age of the bees tested, by the concentration of the chemical used as the stimulus, and by the genotype of the bees tested.

Materials and Methods

For all experiments, caged brood from individual queens was emerged in an incubator during a 24-h period and the young bees placed in glass-fronted wooden cages described by Kulinčević and Rothenbuhler (1973). These cages were arranged several inches apart on shelves in an observation-hive shelter converted to serve as a 35° C walk-in incubator, and testing was performed in place.

The basic testing procedure, adapted from field work by Boch and his associates (1962, 1965, 1971), consisted of the presentation of a measured amount of IPA (mixed isomers, J. T. Baker Co.) diluted in paraffin oil. This solution was inserted under the wire floor of the cage on a small piece of cork. Bees were exposed to both the IPA and the control, paraffin oil alone, for 60 sec. IPA was chosen for testing because it was shown to be a more effective alarm chemical than 2HPT (Boch et al. 1970).

The response of the bees to the IPA had 2 major aspects. The most apparent was a flickering movement of the wings, usually as part of the initial response and continuing intermittently thereafter. The second was a general increase in locomotion, unoriented in direction. Bees made short, jerky runs on the cage surface, ending in brief confrontations with other bees.

The observations which were recorded included the following: 1. *Seconds to react*—the time from insertion of the stimulus until a distinct wing flicker and increase in locomotion was seen. If the bees did not respond, "no reaction" was recorded rather than a time. 2. *Seconds to quiet*—the time at which the activity level returned to that prior to stimulation. This value gave no useful information by itself, but was necessary for calculation of duration. 3. *Duration of the reaction*—a value, in seconds calculated from the first 2 observations, seconds to quiet minus seconds to react. 4. *Initial intensity of the reaction*—a subjective estimate of the strength of the reaction based on both the number of bees involved and the degree of change in activity level. Initial intensity was recorded as none, if there was no reaction, or as weak, medium, strong, or very strong.

Statistical comparisons involving seconds to react and duration of the reaction were made using one-way analysis of variance and least significant difference tests. Intensity of the reaction was analysed by use of chi-square.

Age of Bees

The first experiment was designed to determine the ages at which the bees would give consistent, measurable responses. Testing of 6 cages of 50 bees from each of 3 queens was initiated on the 2nd day following caging and continued until the 13th day. The bees were tested once each day. A second experiment, involving less concentrated IPA as the stimulus, was begun on the 1st day following caging and continued until the 4th day.

Intervals Between Tests

Another concern was the minimum time which must elapse between repeated tests on the same bees to avoid

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such problems as sensory adaptation. A subset of 6 cages from the 18 in experiment I tested to determine the best ages, was retested after 60-min, 30-min, 15-min, and 5-min intervals on days 3, 4, and 5 following caging.

Number of Bees per Cage

To maximize the number of samples obtainable from each queen with the small amounts of brood available, it was desirable to determine the smallest number of bees which would produce clear responses. Using progeny from 1 queen, 3 cages each were made up with 50, 25, 10, and 5 bees, and subsequently tested twice per day.

Concentration of IPA

As the magnitude of the response varied with the strength of the stimulus, an assessment of the concentration of IPA which would give the clearest response was necessary. For this purpose, IPA was diluted in paraffin oil, by volume, to dilutions of 1:2, 1:10, 1:100, 1:1000, and 1:10,000. Twenty-four cages of bees from 4 different inbred lines were tested once a day for 4 days with each of the 5 concentrations of IPA.

Genetic Stock

Our primary goal was to develop a laboratory test which would reflect the different temperaments of various honey bee stocks as evaluated during regular colony management procedures, especially stinging frequency. This was demonstrated using 4 inbred lines which differed in colony defensive behavior, the Brown-Caucasian (Br-Cau) and Van Scoy lines (Boch and Rothenbuhler 1974) and 2 lines selected for resistance and susceptibility to Hairless-Black Syndrome (Kulinčević and Rothenbuhler 1975). All were available at The Ohio State University Bee Laboratory. The Br-Cau and resistant lines were characterized as cross, the Van Scoy and susceptible as gentle. Six cages from one queen of each line were used in testing the various concentrations of IPA.

Results

Age of Bees

Prior to stimulation with IPA, the bees usually were observed moving about the cage on the top, sides, back and floor. Beginning on the 7th day, and occasionally as early as day 4 or 5, the bees exhibited "running" behavior. Bees of this age were photopositive and ran on the glass cage front. When "running" occurred, the bees reacted only slightly, slowly calmed down, or ignored the IPA entirely. Measures of a reaction to the IPA were unobtainable under such conditions. Measurable responses were obtained on days 2–7. As seen in Table 1, seconds to react and duration of the reaction were not significantly different between days in experiment I.

Analysis of the data from experiment II showed that bees tested on the 1st day reacted more slowly (25.8 sec) than on days 2–4 (18.7 sec to 20.6 sec). The duration of the reaction had a less clear-cut relationship, but was about the same for all 4 days. In both experiments, the intensity of the reaction was not affected by age. Thus, to obtain consistent values and to avoid most of the "running", testing should be carried out on days 2–5 following caging.

Table 1.—Effect of age on seconds to react and duration of the reaction. Means (± standard deviation) followed by different letters are significantly different.

Day after caging	Experiment I: Seconds to react	Experiment I: Duration of the reaction (sec)	Experiment II†: Seconds to react	Experiment II†: Duration of the reaction (sec)
1st			25.8±23.2a	39.5±38.3c
2nd	5.8± 5.2	59.5±23.8	20.2±22.0b	33.7±31.0cd
3rd	7.4± 7.5	58.2±19.2	18.7±21.2b	31.8±32.4d
4th	9.5±11.6	60.0±28.3	20.6±20.2b	39.3±36.7c
5th	5.0± 6.2	59.2±19.9		
6th	5.0± 5.3	63.6±20.5		
7th	6.1± 4.8	63.3±19.0		
F	1.5465	1.9073	4.160**	4.290**
d.f.	5 & 70	5 & 70	3 & 357	3 & 357

** $P < 0.01$.
† Experiment II involved more dilute concentrations of IPA in paraffin oil as the stimulus than did Experiment I.

Intervals Between Tests

Observations on the bees that were retested showed that the only interval having a significant effect was 5 min (Table 2). During a retest after 5 min, bees took more than twice as long to react and did so for shorter periods of time. The intensity of the reaction was not affected. An interval of 15 min or more between repeated tests on a cage was sufficient to allow a return to an unstimulated condition. The procedure decided on for further experiments was to allow at least 1 h to elapse between tests.

Number of Bees Per Cage

The cages with only 5 bees showed several signs of aberration. Bees in all 3 cages showed a greater tendency to not react than did the larger groups. One cage population died out by day 3. As seen in Table 3, these bees took 3–5 times longer to begin a reaction than did the bees in the 3 larger groups and stopped sooner. As intensity was assessed partly in terms of the number of bees reacting, these small groups were scored as being very weak in their reaction.

Of the remaining 3 group sizes, 25 proved to be the number for which it was easiest to determine the onset and termination of the reaction. Therefore, 25 bees per cage were used for subsequent experiments.

Table 2.—Effect of length of interval between tests on seconds to react and duration of the reaction. Means (± standard deviation) followed by different letters are significantly different.

	First test	Retests: Length of interval between tests 60 min	30 min	15 min	5 min
Seconds to react	7.7a ±11.1	8.3a ±9.4	9.2a ±8.6	8.9a ±8.0	21.4b ±19.0
F = 10.832**			d.f. = 4 & 87		
Duration of reaction (sec)	60.1c ±27.6	60.0c ±15.3	53.5c ±20.4	53.2c ±17.5	44.5d ±24.9
F = 5.94**			d.f. = 4 & 87		

** $P < 0.01$.

Table 3.—Effect of the number of bees per cage on seconds to react, duration and intensity of the reaction. Means (± standard deviation) followed by different letters are significantly different.

	No. of bees per cage			
	50	25	10	5
Seconds to react	4.2a ±2.6	7.3a ±5.8	7.9a ±4.8	21.8b ±15.0
	F = 14.898** d.f. = 3 & 68			
Duration of reaction (sec)	61.8c ±17.9	52.2cd ±16.6	66.5c ±21.1	37.6d ±19.5
	F = 9.173** d.f. = 3 & 68			
Intensity of reaction (% of observations per category)				
None	6	0	6	38
Weak	0	6	0	8
Medium	11	38	22	31
Strong	72	50	66	23
Very strong	11	6	6	0
	100	100	100	100
	x^2 = 24.036* d.f. = 12			

* $P < 0.05$, ** $P < 0.01$.

Concentration of IPA

As seen in Table 4, mean seconds to react decreased from 41.6 sec to 8.0 sec with increasing concentrations of IPA and duration of the reaction increased from 10.6 sec to 55.8 sec. Mean values of seconds to react and duration of the reaction were not significantly different between concentrations of 1:2 and 1:10, but were different for all other comparisons.

Intensity of the reaction was directly related to concentration of IPA (Table 4). The greatest proportion (55%) of the observations of the response to a dilution of 1:10,000 were judged as no response. For the most concentrated dilution, 1:2, the most frequent evaluation (57%) was a strong response. A difference between the 1:10 and 1:2 dilutions, not evident in seconds to react or duration of the reaction, was seen here. The majority of responses with 1:2 were strong, the majority with 1:10 were medium. However, the 1:10 dilution gave the best behavioral differentiation between lines of bees and was therefore chosen as the best stimulus level for the procedure being developed.

Table 4.—Effect of IPA concentration on seconds to react, duration, and intensity of the reaction. Means (± standard deviation) followed by different letters are significantly different.

	Concn (parts IPA in total, by volume)				
	1:10 000	1:1 000	1:100	1:10	1:2
Seconds to react	41.6a ±23.3	30.8b ±23.1	15.8c ±17.3	9.5d ±8.6	8.0d ±8.7
	F = 76.99** d.f. = 4 & 357				
Duration of reaction (sec)	10.6e ±21.4	20.4f ±24.0	41.8g ±23.7	52.1h ±25.4	55.8h ±26.3
	F = 72.64** d.f. = 4 & 357				
Intensity of reaction (% observations per category)					
None	55	35	11	3	1
Weak	34	42	20	11	3
Medium	11	19	44	64	26
Strong	0	4	23	17	57
Very strong	0	0	2	5	13
	100	100	100	100	100
	x^2 = 295.675** d.f. = 16				

** $P < 0.01$.

Table 5.—Effect of genotype on seconds to react, duration, and intensity of the reaction. Means (± standard deviation) followed by different letters are significantly different.

	Inbred line			
	Gentle		Cross	
	Van Scoy	Susceptible	Resistant	Br-Cau
Seconds to react	30.8a ±23.5	22.0b ±21.3	15.9c ±19.9	16.0c ±20.6
	F = 6.054** d.f. = 3 & 357			
Duration of reaction (sec)	21.1d ±24.1	43.2e ±21.7	43.3e ±24.3	37.0e ±20.1
	F = 5.57** d.f. = 3 & 357			
Intensity of reaction (% observations per category)				
None	25	18	12	12
Weak	15	26	25	9
Medium	25	35	27	16
Strong	32	21	35	25
Very strong	3	0	1	38
	100	100	100	100
	x^2 = 295.675** d.f. = 16			

** $P < 0.01$.

Genetic Stock

Results showed differences between the various stocks which were indicative of their field temperaments (Table 5). The stocks rated as gentle took longer to react (30.8 and 22.0 sec) than did the stocks rated as cross (15.9 and 16.0 sec). For the character of duration, the gentle Van Scoy bees reacted for a shorter period of time (21.2 sec) than did the cross bees (43.3 and 36.9 sec), but the gentle susceptible bees were similar to the cross bees (43.2 sec). Finally, when comparing the intensity of reactions, the 2 gentle lines and the cross resistant line were similar in having the majority of responses as medium or weaker, although the Van Scoys had the greatest frequency of no reaction. The strongest reacting bees were the cross Br-Cau, with 63% of the responses strong or very strong.

Discussion

Under normal conditions, the alerting and defense of the colony is undertaken by bees who have just reached foraging age, generally 2–3 weeks old (Free 1965). The caged, newly emerged bees used in this study showed sufficient response to the IPA component of alarm pheromone to justify their use in measuring alarm behavior. In fact, as bees less than 1 week old produce little or no IPA and bees 2–3 weeks old show maximum amounts (Boch and Shearer 1966), the use of newly emerged bees should reduce the contamination of the test by naturally occurring alarm pheromone. Additionally, although young bees reacted consistently to the IPA from 2–7 days after caging, the increasing occurrence, with age, of "running" prevents the use of this procedure for testing bees older than 5 or 6 days. The response observed included partial extension of the wings and increased locomotion. Later stages of the behavior pattern, i.e., sting extrusion and attack, were not seen. This

agrees with observations of colony responses to IPA (Boch et al. 1971) in which additional stimulation, such as movement, was required to elecit stinging.

The nature of alarm communication is such that the chemicals involved must reach sufficient concentration to cause a reaction and then disperse again within a relatively short period of time. This is necessary to sharply localize the source of the stimulus. It is also adaptive for the organisms involved to recover quickly from stimulation by an alarm chemical, so that they can respond effectively to a second event. As indicated by this test, young bees will respond again as quickly within 15 min after an initial stimulation. This is useful for a testing procedure because it allows repeated observations of the same bees to be made in a relatively short period of time.

As bees are not seen to exhibit alarm behavior at such non-hive locations as feeding sites (Michener 1974), a very small number of individuals in a cage might provide insufficient cues to define the group as a colony to be alerted and defended. Whether the abnormal performance of groups of 5 was due simply to too few bees to find and utilize the available water and sugar syrup, or to lack of more esoteric social requirements, was not determined.

The strength of the stimulus affected all 3 aspects of the behavior which were measured. Increasing concentrations of IPA were associated with quicker, longer-lasting, and stronger reactions. This might lead one to suppose that bees expressing higher levels of defensive behavior might do so because they release greater concentrations of alarm pheromone. Boch and Rothenbuhler (1974) and Kerr et al. (1974) found that IPA production was not correlated with the expression of defensive behavior. However, Kerr et al. did find a correlation of the behavior with 2HPT. Crewe (1976) reported that the levels of IPA and 2HPT in *A. m. adansonii,* a notoriously defensive race of bees, fell within the ranges reported for less defensive Canadian populations (Boch and Shearer 1965, 1966).

An alternative hypothesis proposed by both Boch and Rothenbuhler (1974) and by Crewe (1976) is that the difference in behavior reflects differences in the threshold of response. This is consistent with the relationships expressed in Bossert and Wilson's (1963) model of olfactory communication, which indicates that variation of the time factor is dependent on variation in K, threshold density, as all other model variables are constant in the procedure used here.

Samples of bees from inbred lines that showed different temperaments during colony manipulations gave responses to the IPA that were consistent with the field evaluations. Thus, this experimental procedure could be used as an effective tool in selection of defensive behavior variants, particularly if it is correlated with quantitative measures of whole colony defensiveness. There are 2 additional aspects of the laboratory test which make it more desirable for behavior genetic studies than field tests of whole colonies. Emergence of brood in an incubator provides more control of the genetic make-up of the sampled population by eliminating drifters. Any colony in an apiary will contain some proportion of bees which have drifted in from another colony, and come from a different mated queen. Also, using the small samples makes it possible to evaluate a newly laying queen without having to wait 6 weeks or more until her colony is populated by only her offspring.

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