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Behavior of Workers of *Solenopsis invicta* (Hymenoptera: Formicidae) to the Queen Recognition Pheromone: Laboratory Studies with an Olfactometer and Surrogate Queens[1]

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ABSTRACT

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Red imported fire ant (RIFA), *Solenopsis invicta* Buren, queens produce a queen recognition pheromone that attracts daughter or conspecific worker ants singly or in groups. Their attractiveness ceases if they are separated from their colony for more than 30 min, but returns immediately when they are reunited with their workers. Whole-body queen extracts of the pheromone are attractive when carried by an air stream into an olfactometer or when applied to surrogate queens (pieces of rubber septa). Extracts of the pheromone induce some attraction at a concentration of 0.01 queen equivalent, with maximum response at concentrations of 0.5 to 5.0 queen equivalents. The response of workers to extracts of other RIFA castes was significantly less than that to queen extracts in all bioassays.

The production by ant queens of a volatile pheromone that attracts their workers was postulated in the early 1900s by Wheeler (1910). Subsequently, several investigators have used a variety of methods to demonstrate the existence and attractiveness of such a pheromone. For example, Stumper (1956) demonstrated attraction of ether and alcoholic extracts of queens of *Lasius alienus* Foerster and *Pheidole pallidula* Nylander applied to pieces of filter paper, sponge, or elder pith, and Watkins and Cole (1966) showed that workers of the genus *Neivamyrmex* and *Labidus* spp. were attracted to squares of a paper grid on which a queen had been confined. Brian (1973), working with *Myrmica rubra* L., measured the influence of queens by the effects produced on brood and concluded that queens communicate their presence to workers both by chemical and topographical stimuli. Jouvenaz et al. (1974) confined queens of *Solenopsis geminata* F. and *S. invicta* Buren on individual squares of a grid on filter paper and observed that the number of workers attracted to these squares was greater than to any other square. Glancey (1980) demonstrated the existence of a queen "recognition" pheromone in *S., invicta* by applying extracts of queens to filter paper strips and small cylindrical pieces of applicator stick; and Vander Meer et al. (1980) discovered that the poison sac of the queen was the source of a pheromone attractant. Subsequently, Glancey et al. (1981) found that artificially dealated virgin queens also produce the pheromone.

We recognize from the studies cited, as well as others, that the queen produces several pheromones that are used to regulate and integrate the communal activities of the colony. For the purposes of this paper, however, we have utilized the term "queen recognition pheromone" to describe the behavior elicited by crude extracts of *S. invicta* queens or live queens, even though a wide variety of responses may be affected, including attraction, recognition, and tending of the queen. Here, we report on laboratory studies of the behavior of workers to the pheromone, using an olfactometer and pheromone-treated surrogate queens.

Materials and Methods

Source and Maintenance of Test Insects

All ants for laboratory tests (except the field queens) were obtained from colonies maintained in Williams cells (Bishop et al. 1980) and fed honey-water and Banks diet (Williams et al. 1980). With the exception of a few colonies that were maintained in a screen cage outdoors under ambient conditions (July-August), all of the colonies were reared in laboratory rooms at 27 ± 1°C. The outdoor colonies were used only in a few preliminary tests to study the effect of ambient environmental conditioning.

Queens for extraction of queen recognition pheromone were obtained by: (1) capturing dealated mated or virgin females from field colonies; (2) collecting newly mated queens after mating flights and holding them 2 weeks in the laboratory to allow time for initiation of pheromone production; or (3) collecting alate virgin queens, causing them to dealate by confinement in small vials, and holding them for 2 weeks for pheromone production (Glancey et al. 1980).

Worker ants (responders) used in these tests were taken directly from a Williams cell that contained brood and placed in a small medicine cup, the sides of which had been dusted with talc. Other studies at our laboratory have shown that the youngest workers tend the brood and queen and are more responsive to the pheromone than older foraging workers (unpublished data). Just before an experiment, the desired number of ants for each test were collected from the cup and released in the test arena.

Chemical Extractions

The extracts were prepared by allowing the queens or other castes to soak for a few hours in benzene and then crushing them under solvent with a glass tissue grinder. (Prior observations indicated hydrocarbon solvents were suitable for extraction of the pheromone from whole

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bodies of queens.) Next, they were filtered over anhydrous Na_2SO_4 and through silanized glass wool (contained in a disposable pipette). Concentrations were adjusted to 100 mg of body weight equivalent per ml. Extracts of the various castes were tested both on the basis of the amount of material extracted for equivalent body weights (2 mg per test) and the amount extracted per individual ant (0.2 ant per test).

Olfactometer Design and Test Procedures

The basic olfactometer consisted of a Wilson cell (Wilson 1962) without a lid, in which worker ants responded to the queen recognition pheromone as it was introduced in air diffused through a cotton swab. In our initial studies, we used either a 9- or 19-cm-diameter Wilson cell (See Fig. 1). Two of the entrance-exit ports were sealed leaving two openings at 180° to each other. To avoid problems with static electricity, we covered the floor of the cell with a 1-cm layer of Castone (Ranson and Randolph, Toledo, Ohio) which was wetted with several ml of water just before each test. Live queens were tested by confining them in a Pasteur pipette with small pieces of plastic screen. One-half of a hollow plastic-stem cotton swab (Johnson and Johnson, Skillman, N.J.) was placed over the narrow end of the pipette and the swab was inserted into one of the ports in the Wilson cell. The area around the pipette stem was plugged with clay, and the walls of the cell were dusted with talc to prevent escape of the ants. The pheromone was conveyed into the olfactometer by passing medically pure, compressed air through a plastic tubing into the pipette

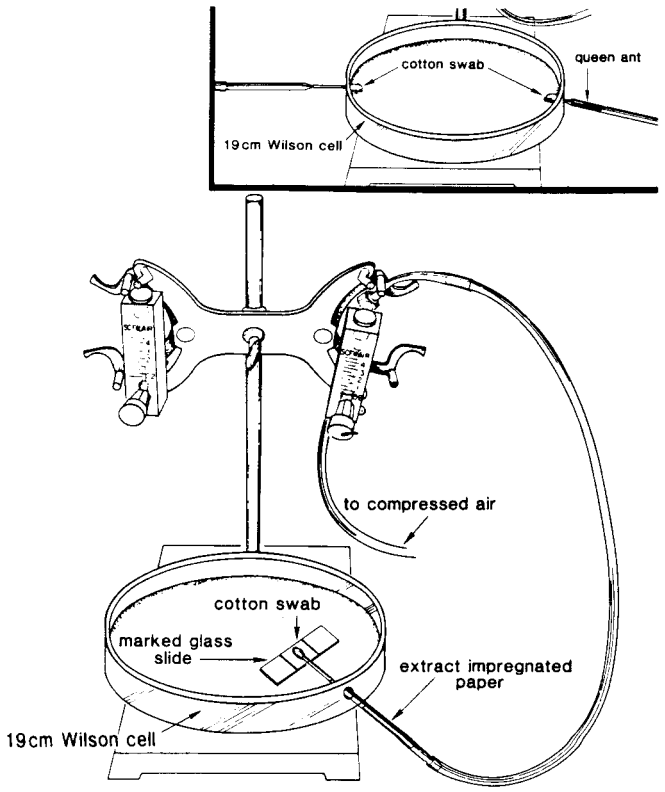

FIG. 1.—Design of one-port and two-port (inset) olfactometers. Sides of dish arena were coated with Fluon to prevent escape of ants.

which contained the queen or extract. The airflow was controlled (0.5 liters/min unless indicated otherwise) with a series of flowmeters. An identical air flow device (without queen or extract) was inserted in the opposite port of the olfactometer.

At the start of each test the responder ant or ants were confined for a few minutes under an inverted medicine cup in the middle of the Wilson cell. After release they could approach either of the ports and cluster around the swab (Fig. 2). Three different tests were conducted with live queens and the different castes. In the first test the response of one responder or groups of 20 responders was observed. When one responder was used, we determined the total time spent at either port over a 10-min period; when 20 responders were used, we counted the total number of responders in each port at intervals of 3, 6, 9, 12, and 15 min. Four or five replicates were conducted in each test series.

In the second test we evaluated the attractiveness of queens at different time intervals after removal from the the colony, and in the third test the attractiveness of a queen was compared with that of other castes. The latter was done by confining a colony queen in one pipette of the olfactometer and 20 larvae, five workers, or an alate in the other pipette. Twenty responder workers with four to five larvae or pupae were used in each of these tests. Responders from the same and alien colonies were assayed.

Tests with extracts of queens and the various castes were evaluated in an olfactometer that was modified by closing three of the exit-entrance ports (see Fig. 1.) Extracts to be evaluated were pipetted onto small strips of filter paper (0.3 by 3.0 cm). After evaporation of the solvent (0.5 min), the paper was placed inside the Pasteur pipette and a hollow-stem cotton swab was placed over the end of the pipette. The swab was inserted into the cell a distance of about 2.5 cm and rested on a glass microscope slide (see Fig. 1). The slide was used to minimize contamination of the Castone with pheromone. Twenty responder ants and four to five larvae or pupae were used per test.

The reaction of the responder ants was scored by counting the number of ants in a 2-cm^2 area around the swab at 1-min intervals for 5 min (Fig. 2). The sum of the five counts constituted one trial. This counting procedure was used because of the typical behavioral response of worker ants to their queen. Ordinarily, workers lick, groom, and feed the queen and collect the eggs she lays; thus, there is a constant flow of workers to and from the queen. In our tests, workers responded to the pheromone and sometimes clustered around the cotton swab, but not receiving other queen stimuli they repeatedly left the count area and then returned. Because of the continual movement in and out of the count area, a series of counts gave the most accurate tally of the response of the workers. A solvent control was tested in each set of experiments.

After completion of each test, the pipettes and swabs were discarded and the cells and slides were washed with hot soapy water and rinsed with acetone. Six to 10 replications were made.

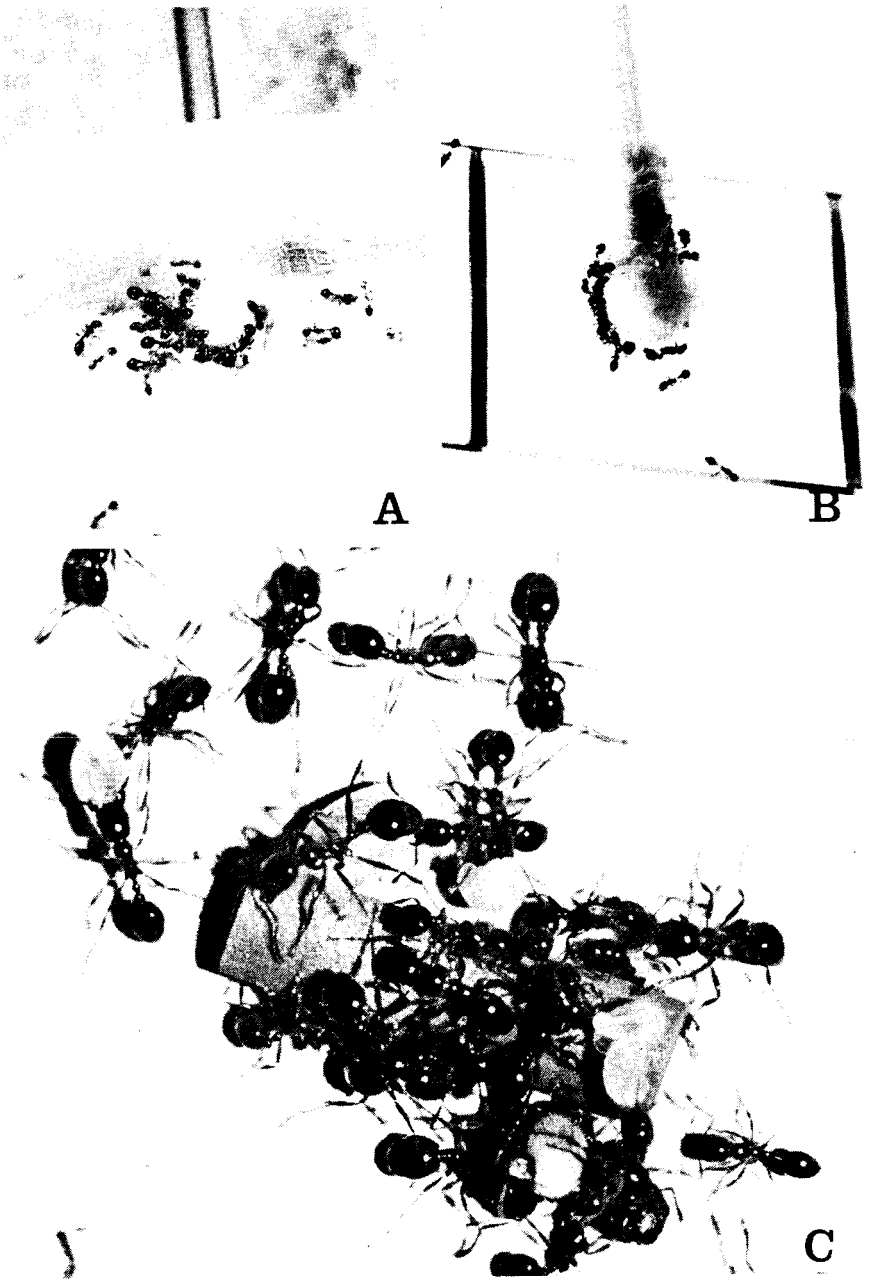

FIG. 2.—Worker ants attracted to queen recognition pheromone released in two-port (A) and one-port (B) olfactometers and to surrogate queen (C). Note that workers placed brood on or near surrogate queen, a response that is also noted toward mother queens placed outside their nest.

Two tests were conducted with this olfactometer to (1) evaluate the response of workers to crude extracts of the different castes comparing concentrations of pheromone based on extracts from equivalent body weights or from equal numbers of total ant bodies, and (2) determine the response to various concentrations of the pheromone in terms of the activity induced by queen equivalents (QE), i.e., dilutions of the average amount of pheromone extracted from a single queen.

Surrogate Queen Tests

Since the worker ants normally are attracted to the queen by the queen recognition pheromone and thus interact with her directly, we devised a surrogate queen to study this behavior. Initially, we used 1-cm lengths of wooden applicator sticks; however, we found later that rubber needle-seal septa (Chemical Research Supplies, Inc. Addison, Ill.) were more suitable. Eight-millimeter sections of the septa were quartered lengthwise, extracted with methanol, methylene chloride, and hexane, and then dried at ca. 80°C for several hours before use. The sections of septum were about the size of a physogastric queen (8 by 3 mm; ca. 20 mg). Septa were placed in a given amount of the extract in solvent (usually 20 to 30 μl) in a sealed vial. At the time of the test, the septum was removed and allowed to dry for 15 to 30 min. The bioassay chamber consisted of a Wilson cell (9 cm) arena similar to that used in the olfactometer bioassays with all of the exit-entrance ports closed. An observation square (2 cm^2) was drawn in the center of the cell. A septum or surrogate queen was placed in the

observation square. Next, 20 workers with a small amount of brood (usually three larvae or pupae) were introduced. Untreated septa were always ignored by the workers; however, their response to pheromone-treated septa involved attraction, licking, movement of septa, and deposition of brood near the septa. We quantified the data simply by counting the numbers of ants clustering in the 2-cm^2 area about the septum at 1-min intervals for 5 min (Fig. 2).

Two sets of tests were conducted. In the first, the response of workers to crude extracts of the different castes was determined by comparing extracts of equivalent body weights or extracts from equal numbers of individual ants; in the second test, the response to different concentrations (QE) of the pheromone was determined. The response of workers to solvent-treated septa was evaluated in each test series.

Statistical Procedure

Data for all of the tests except those in which dose-response relationship was determined were evaluated for significance by analysis of variance or Duncan's multiple range test. All dosage-response data were corrected for control responses by Abbott's formula and analyzed by linear regression, using the arcsin transformation of the percent response and the log of the dose.

Results and Discussion

Response to Live Queens and Other Castes

The response of individual worker ants to the queen recognition pheromone of their mother queen was observed in the small (9-cm) olfactometer (air flow of 0.25 liters/min). Data from 10 individual workers from each of five colonies maintained inside or outside the laboratory show that there was a statistically significant difference ($P < 0.05$) between the total time spent at the port where the pheromone was released (190 ± 14.6 sec) and that spent at the control port (98 ± 11.3 sec). Also, the analysis showed that the differences in attraction were the same for both preconditioning environments.

The same experiment was repeated with individual workers tending a larva. The time spent by the larva-carrying worker in the control port averaged 114.2 ± 10.2 sec and in the queen port, 140.9 ± 15.9 sec. The difference between the means was not significant. There was no noticeable difference between the responses of ants maintained outdoors vs. indoors.

The reason for the lack of response by larva-carrying workers appeared to be related to an inability to detect or respond to the pheromone while carrying the larva. When no larva was present, orientation by the workers to the queen air stream was rapid and movement to the queen port was very direct. If the ant went to the control port, it generally stayed a few seconds and then began searching again until it oriented to the queen air and visited the queen port. The overall tendency was to remain in or around the queen port. We found that the reaction of a larva-carrying ant was much different. The ant often appeared confused, moving in and out of the pheromone air stream but never orienting directly to it. If the ant located the control port without detecting the pheromone, it would settle there, often remaining for the entire length of the test. If it located the queen port, the ant became excited, running to and away from the cotton swab. At other times, the ant did not find either the queen port or the control port and simply rested alongside the wall of the cell.

An interesting behavioral pattern was displayed by the non-larva-carrying ants when they detected the pheromone. They raised their thoraces and heads by standing on the second or third pair of legs and then extended their antennae straight up in the air and vibrated them. After such a display, the ants moved to the queen port. However, the larva-carrying ants had difficulty raising their thoraces and heads, and the antennae were constantly touching the larva. Very seldom were ants seen to stop antennating the larvae and begin the "queen orientation" waving. Lack of response to the pheromone could have resulted from this apparent "inability" to raise their antennae and orient to it.

Although the prior tests showed that single workers are attracted to the queen recognition pheromone, a single ant in an alien environment might behave differently in the presence of other ants. Therefore, we also tested groups of ants (20) supplied with four or five larvae or pupae. The analysis of variance of four replicates showed that the difference between means of ants attracted to the queen port (38.0 ± 3.3) and those attracted to the control port (11.5 ± 2.3) was significant ($P < 0.01$). There was no difference in the response of ants maintained inside and outside the laboratory.

One of the observations made during these tests was that if some ants carried brood to the control port, other ants which had found the queen port would actively recruit these workers to carry the brood to the queen port. Also, with more ants in the test, there seemed to be an aggregation effect; i.e., as more ants arrived at the queen port, fewer ants left. To avoid bias from this behavior, we did not make observations for more than the first 15 min of the test.

The effect of isolation of the queen from her colony on the response of her workers was tested by separating her from her workers for 1, 2, or 3 h. The data revealed that there was no difference between the response to the queen and to the control over the times of isolation used in this test. As a result, a second test was conducted, in which each of three queens were placed individually in the pipette of an olfactometer and air was blown over them for 0, 15, 30, 45, and 60 min. At the end of each time interval, a separate set of responder ants were released in the olfactometer arena. Data from tests with the three queens revealed that they were not attractive after 30 min. The average number of responders attracted at the first three time intervals (0 to 30 min) ranged from 23 to 29, compared with 12 and 14 after 45 and 60 min, respectively. The differences between the first three and last two time intervals were significant ($P = 0.05$). Workers rapidly responded to their queen

when she was returned to their colonies after more than 30 min of separation, indicating that pheromone release by the queen is dependent on contact or close proximity to workers. This also negates the possibility that the workers produce the attractant material and apply it to the queens.

The data (Table 1) in the test comparing attraction of workers to volatiles of live queens and other castes showed that the queens were more attractive than larvae, workers or female alates.

Response to Extracts of Queens and Other Castes

Our first test comparing the attractiveness of extracts of different castes showed that extracts of queens were significantly more attractive ($P = 0.05$) than extracts of the other castes in both the olfactometer and surrogate queen bioassays (Tables 2 and 3).The greatest attraction to the queen extract occurred in the surrogate queen test (ca. threefold greater than for major workers); however, since crude extracts were used and the workers could contact the surrogate queen directly, added attraction may have resulted from an aggregation effect or from other nonvolatile semiochemicals in the extract, such as the cuticular or postpharyngeal gland hydrocarbons (Thompson et al. 1980).

Except for data from the major worker extracts, the surrogate queen data, whether based on body weight or ant equivalents, were similar for each caste. Again, the differences between the major and the other castes may reflect attraction by other chemicals (e.g., trail pheromone), since the body weight sample contained the equivalent of three major workers.

In the surrogate queen tests, a few larvae and pupae were placed in the test chamber with the worker ants. The workers often placed the brood by the surrogate queen treated with the queen recognition pheromone, but not with surrogates treated with extracts of other castes. This behavioral pattern also occurs when a queen and brood are placed outside the nest, indicating that it is a behavior unique to worker-queen interactions.

The linear regression analysis of the concentration-response data is plotted in Fig. 3 and 4 with the 95% fiducial limits. The response of the workers to the pheromone was significant by concentration for both the olfactometer ($F = 87.4$; $P = 0.001$) and surrogate queen ($F = 92.1$; $P = 0.0001$) bioassays. Some activity was noted at 0.01 QE, but maximum response occurred at 0.5 to 5.0 QE.

Imported fire ant queens produce a volatile queen recognition pheromone that can be extracted with hydrocarbon solvents and which attracts individual or groups of worker ants. The worker ants respond to the pheromone, whether it is released into an olfactometer or impregnated on a surrogate queen (a rubber septum). The overall behavior appears to follow the sequence of recognition, attraction, and tending. The latter involves a variety of responses when the workers make contact with the queen directly; however, the responses to surrogate queens involve primarily licking, brood deposition, and movement of the surrogate. Continuous release of the queen recognition pheromone appears to be dependent upon direct contact between the queen and her workers as evidenced by the fact that queens lose their attraction when held in a column of air for ca. 30 min and quickly regain it when returned to their colony. Worker ants respond equally well to pheromone released by their mother queen and alien queens. No other caste produces a pheromone that induces similar behavioral responses. Although not reported in this paper, other tests have shown that response to the surrogate queen by workers is very different from that toward surrogate treated with a food (soybean oil).

The worker response to pheromone extracts in the two types of bioassays is evident at 0.01 QE and plateaus at 0.5 QE. Since the tests were conducted with whole-queen extracts, we cannot determine at this time whether the extract is composed of one or more than one pheromones.

Successful isolation, identification and synthesis of the active pheromonal components will provide a useful tool for increasing the attractiveness and, possibly, species specificity of toxic baits.

Table 1.—Comparison of the attraction of worker (responder) ants when exposed simultaneously to two air streams one of which was passed over a queen and the other over a female alate, larvae, or workers

Test caste	Source of responder ants	Total no. of responder ants attracted to:		Difference[a]
		Queen	Test caste	
Larvae	Same colony	42.2	18.0	24.2
	Alien colony	44.2	22.2	22.0
Workers	Same colony	25.0	9.4	15.6
	Alien colony	39.8	14.0	25.8
Female alates	Same colony	32.2	8.4	23.8
	Alien colony	26.6	4.0	22.6

[a]Differences between queen and test caste were significant (analysis of variance) in all cases at the $P = 0.05$ level. The numbers of responder ants attracted, whether from the same or an alien colony, were not significant.

Table 2.—Response of workers of the red imported fire ant in an olfactometer to crude benzene extracts of workers and mature and immature sex forms

Extract	Mean response to solvent extractions based on [a]: Equivalent body wt[b]	Equivalent no. of ants extracted[c]
Sex larvae (♀)	4.4a ± 1.1	3.1a ± 1.6
Sex pupae (♂)	5.4a ± 1.9	3.5a ± 1.5
Minor workers	5.6a ± 1.6	11.6b ± 2.6
Major workers	10.9ab ± 1.9	10.4b ± 2.0
Male alates	7.6ab ± 3.2	7.0ab ± 1.8
Control (benzene)	8.6ab ± 2.5	8.6ab ± 2.5
Female alates	14.6b ± 3.5	8.5ab ± 1.4
Queens (field collected)	27.6c ± 1.9	27.6c ± 1.9

[a]Total number of responder ants attracted: average of eight replicates. Means followed by same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).
[b]Extract from 2 mg of body weight.
[c]Extract from 0.2 ant.

Table 3.—Response of workers of the red imported fire ant to surrogate queens treated with benzene extracts of workers and mature or immature sex forms

Extract	Mean response[a] based on: Ant equivalents	Body wt
Sex larvae (♀)	8.6a ± 2.5	5.9a ± 1.2
Sex pupae (♀)	5.5a ± 1.4	5.8a ± 1.5
Minor workers	6.5a ± 1.4	9.4a ± 2.8
Major Workers	11.5a ± 2.3	18.4b ± 3.9
Alates (♂)	7.9a ± 1.7	7.9a ± 1.6
Alates (♀)	8.9a ± 2.5	11.0a ± 3.0
Control (benzene)	4.8a ± 1.5	4.8a ± 1.5
Queens (field collected)	46.5b ± 5.1	46.5c ± 5.1

[a]Total number of ants attracted to the surrogate queen: average of eight replicates. Means followed by the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

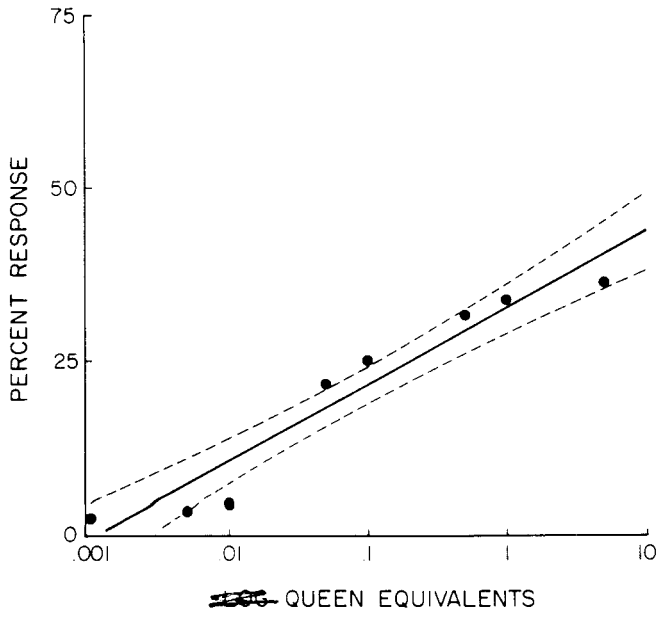

FIG. 3.—Mean response of RIFA workers to varying concentrations of a crude extract of the queen recognition pheromone in the olfactometer bioassay.

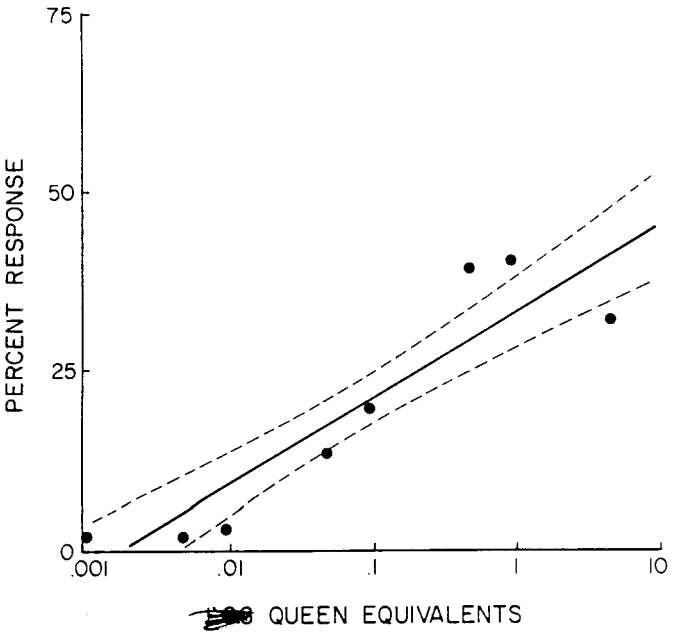

FIG. 4.—Mean response of RIFA workers to varying concentrations of a crude extract of the queen recognition pher-

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