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BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES WITH LIVE LARVAE AND LARVAL RINSES OF THE RED IMPORTED FIRE ANT, *Solenopsis invicta* BUREN (Hymenoptera: Formicidae)

B. MICHAEL GLANCEY[1] and JOSEPH C. DICKENS[2]

[1]*USDA, ARS, Insects Affecting Man and Animals Research Laboratory Gainesville, Florida*

[2]*USDA, ARS, Boll Weevil Research Laboratory Mississippi State, Mississippi*

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Abstract—Behavioral and electrophysiological studies with live intact larvae and larval rinses of the red imported fire ant, *Solenopsis invicta* Buren, give undeniable evidence of a volatile material associated with the larvae of the ant that is capable of eliciting a response from brood-tending workers. In a Y-tube bioassay, worker ants were attracted equally to an airstream blown over sibling larvae or heterocolonial larvae. Workers were also attracted to a rinse of the larvae in a spot bioassay, aggregated about a piece of surrogate brood in another bioassay, and retrieved surrogate brood treated with the rinse material. A dose-response curve constructed from electroantennograms of workers revealed a receptor response of 1–100 brood equivalents.

Key Words—*Solenopsis invicta*, Hymenoptera, Formicidae, brood recognition, pheromone, fire ant, behavior, olfaction, orientation, bioassays.

INTRODUCTION

The brood of an ant colony can be considered to be not only the growing point of the colony, but also the storage tissue or energy capital (Wilson, 1971). When the colony falls upon hard times, the brood can serve as the prime source of food. It is not surprising then that the early investigators of the behavior of workers toward the brood focused mainly on the trophallaxis of either the stomodeal or proctodeal larval secretions (Stager, 1923; LeMasne, 1953; Torossian, 1961). While these studies did explain some of the feeding behavior, other

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behaviors toward the brood were left unexplained. Watkins and Cole (1966) presented the first evidence that the brood of the army ant, *Neivamyrmex apacithorax* (Emery), produced a pheromone that induced specific worker tending behavior. Later experiments by Glancey et al. (1970) showed that the brood of the red imported fire ant (RIFA), *Solenopsis invicta* Buren, also produce a pheromone that elicits particular worker behaviors (e.g., tending, grooming, moving, etc.) and is extractable and capable of being bioassayed. Robinson and Cherrett (1974) found that hexane would remove a behaviorally active material from larvae of *Atta cephalotes*, but they were unable to isolate the active fraction.

Investigations by Walsh and Tschinkel (1974) on RIFA led them to question the report of Glancey et al. (1970). Ignoring the food bioassays conducted by Glancey et al. (1970), they insisted that the latter's findings represented a food response. Walsh and Tschinkel (1974) concluded that they had found the brood pheromone, that it was a nonvolatile contact pheromone, that it was intimately tied up with the cuticle, and that the cuticle had to be contacted in order for a response to follow.

Later, Bigley and Vinson (1974) reported that the brood pheromone of RIFA was triolein, a triglyceride. This finding was questioned by VanderMeer (1983), who did not feel confident about assigning a structure based only on thin-layer chromatography.

This present paper presents the results of additional behavioral studies. The first evidence of a noncontact component of the brood pheromone is presented. Using larval rinses, we assayed workers for contact-mediated and non-contact-mediated responses to the brood pheromone. Additionally, EAG responses of brood tenders to larval rinses were measured.

METHODS AND MATERIALS

Source and Maintenance of Test Insects

Worker ants that were observed to be tending brood were collected from the vicinity of various laboratory colony brood piles. These "brood tenders" are the youngest workers and are more responsive to various pheromones (Glancey, unpublished data) than the older foraging workers. The colonies were maintained at 27 $\pm$ 1°C in Williams cells (Bishop et al., 1980) and fed honey-water and the Brooks diet. This diet, developed by T. Brooks, University of Georgia at Athens, consists of ground beef, peanut butter, eggs, sugar, salt, sorbic acid, vitamins, and water. The materials are blended with gelatin, allowed to cool and congeal, and the solidified material is cut into 1-inch squares. These squares are dipped into melted paraffin (Paraplast). When the test protocol called for the use of live larvae, the larvae were collected from colonies other than the

ones used for the collection of the brood tenders. Field larvae were collected by digging up a field colony and separating the brood from the soil.

Chemical Stimuli

Larval Rinses. The larval rinses for the behavioral bioassays were obtained by collecting field colonies and separating the larvae from the soil and workers. This separation was greatly facilitated by moving the larvae back and forth with the use of a camel's hair brush. This motion caused the larvae to cohere together due to the presence of larval hooks. These larvae could then be lifted away from the pupae. Groups of live larvae were weighed, the numbers counted, and an average weight of 0.53 mg/larva calculated. Only worker larvae were collected, mainly third and fourth instar. An estimated 250,000 larvae were collected in this manner. The larvae were rinsed in nanograde pentane for 30 sec, the extract transferred to a freezer, and the larvae discarded. The rinse was held in a freezer until used in a given bioassay.

In order to ensure that the responses obtained were not due to a food reaction, we compared the response to larval rinses with responses to pentane rinses made from American cockroaches (*Periplanata americana*). The cockroaches were obtained from the USDA's IAMARL cockroach-rearing facility located at the Gainesville Laboratory. Cockroaches, if available from the rearing section, are normally fed to our ant colonies as a source of insect protein. Previous work had shown that worker ants are attracted to and masticate a spot on filter paper to which a cockroach rinse has been applied (Glancey, unpublished data). The roaches obtained from the rearing section were weighed and rinsed with nanograde hexane. Initial tests made with the larval rinses showed that it required 500 larvae (226 mg) to elicit a response from the workers. Accordingly, a solution was made up which gave us 500 larval equivalent (LE) per 20 μl of pentane. Similarly 226 mg of roaches were rinsed in pentane and reduced to 20 μl. Both the brood rinse and the cockroach rinse were quantified by capillary GC with an external standard to give comparable amounts of extracted material (Kovats index range 900–4500) in both rinses.

Electrophysiological Experiments. Volatiles were collected from worker larvae by rinsing 1, 10, or 100 larvae in nanograde pentane for 30 sec. Excess pentane was evaporated under a stream of nitrogen until a final volume of 10 μl was reached. The 1-hexanol (>99% purity) used as a standard was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin).

Bioassays

Attraction to a Spot. A 5.5-cm piece of Whatman No. 1 filter paper was divided into four quadrants by drawing two intersecting lines through the center. A small pencil dot was placed in the middle of each quadrant. A number between

1 and 4 was assigned at random to each one of the dots. Treatments assigned randomly to the dots were 20 μl each of the larval rinse, the cockroach rinse, and the pentane solvent; a fourth treatment consisted of a cohort of live larvae (ca. 300) that were placed on the spot and removed after 15 min. After the larvae were removed, the other dots were treated. The treated filter paper was placed in the bottom half of 5.5-cm plastic Petri dish. Twenty brood tenders obtained from our laboratory colonies were placed in the disk and another dish inverted over the bottom half to keep the ants from escaping. At 1-min intervals, counts were made of the ants within 0.5 cm of the spot.

Olfactometer Bioassay. Live larvae were tested for the production of volatiles in a Y-tube olfactometer. This olfactometer was modeled after a design by VanderMeer et al. (1979) and is described completely by VanderMeer et al. (1988). The olfactometer consists of two 24/40 ground glass joints sealed to one of the arms of a 5-cm Y tube such that 1 cm of each Y-tube arm extended through half the ground glass joints. Three hundred third- and fourth-instar colony or field larvae were placed in one of the choice chambers. The other choice chamber was left blank. Compressed air was split into two streams, each stream being independently controlled by flowmeters. Airflow was regulated at 0.2 l/min into each choice chamber. Fifty brood tenders, selected at random, were chilled for 10 min (6°C) in the refrigerator and then placed in a small bronze wire cage. The open end of the cage was attached to the stem of the Y tube and the airflow turned on.

The initial choice of the first 20 ants that responded by walking into one of the choice chambers was recorded. Ants that were not trapped in the choice chamber and returned to the stem entrance were not counted. The entire olfactometer was rinsed after each test with acetone and dried. Another set of larvae from the same colony was tested with another set of workers from the same colony. However, the choice chamber in which the larvae were placed was reversed. After this test was run, the two scores were combined to give one replicate. This type of procedure eliminated any bias that was inherent in the individual choice chambers. Data were analyzed statistically by use of a chi-square test.

Surrogate Bioassay. This bioassay was developed in our research of the queen recognition pheromone (Lofgren et al., 1983). A section of a rubber needle-seal septum was treated with one of the rinses or pentane. The septum was air dried and then placed in a 9-cm Wilson cell which had all the ports sealed. The septum was placed in a 2-cm^2 area drawn upon the castone bottom of the cell, and 20 worker ants were placed in the cell. The data were quantified by counting the numbers of ants clustering in the 2-cm^2 area at 1-min intervals for 5 min. At the end of the 5-min run, the ants and septum were discarded and a new cell used for the next trial. Three different colonies were tested in this manner for three replications.

Retrieval of Brood Surrogates by Disrupted Colonies. This particular

bioassay was also designed for use in our green pheromone research. When a colony is opened and the brood and workers are scattered about the colony area, the workers immediately begin to collect the brood, lay trails back to the nest, and carry the brood along these trails. We have simulated this in the laboratory by using a laboratory colony in a large box (1.2 m^2) (Glancey et al., 1983). The rearing cell containing the colony is opened and the brood and workers scattered about the box. In the present bioassay, small pieces of colored construction paper (2 mm^2) (Union Camp Corp., Chamblee, Georgia) were soaked overnight in the rinses (larval or cockroach) or solvent. The papers were air dried, the colonies disrupted, and 10 pieces each of the various colored papers deposited in the area of disruption. Differently colored papers were used for each treatment, but no colonies received the same colored paper treated with the same rinse. One hour after the papers were deposited, observations were made of their fate. If the papers were taken inside the cell and placed with the brood, the test was scored as a positive response. Sometimes the ants had difficulty getting the paper through the small entrance hole into the cell. If the ants placed the papers beside the entrance hole because they could not get it into the cell, and if they placed some of the scattered brood next to the papers, then that test was recorded as a positive one. Five replications using five different colonies were made in this manner.

Data for the attraction and surrogate bioassays were evaluated for significance using the general linear model procedure of SAS Institute (1982) and by the Waller-Duncan *K*-ratio *t* test.

Electrophysiology Procedures. Electroantennogram (EAG) techniques utilized in these studies were modified after an earlier study (Schneider, 1957) and are described in detail elsewhere (Dickens and Payne, 1977; Dickens, 1981). In brief, Ag–AgCl capillary electrodes filled with physiological saline (Pantine, 1948; Oakley and Schafer, 1978) were used. The recording electrode was introduced into the distal end of the terminal antennal segment which was prepunctured by a sharpened tungsten needle. The indifferent electrode was inserted into the head capsule. The signal was amplified 10-fold by a Grass P-16 DC microelectrode preamplifier prior to viewing on a Tektronix 5223 digitizing oscilloscope. An *x–y* plotter recorded EAGs on graph paper for subsequent analyses and storage.

Odorous stimuli were delivered on filter paper (8 × 18 mm) inserted into glass cartridges (80 mm long; 5 mm ID) oriented toward the preparation from ca. 1 cm. Stimulus duration was 1 sec with an airflow of 1 m/sec. A range of concentrations was used to develop a dose–response curve. The stimuli were presented in order from the lowest to the highest concentrations. A 3-min interval was allowed between each stimulus. Three replications were run using three different insects. Response to the pentane control was subtracted from response to the other compounds.

Stimulation with 1-hexanol, a component of the green leaf volatile com-

plex (Visser et al., 1979), at 100 μg was used as a standard so that responses from different preparations could be compared (Dickens, 1984; Dickens and Boldt, 1985). In addition, the 1-hexanol is ideal as a standard since most insects, indeed most animals, encounter this odor in their daily environment. Six-carbon alcohols and aldehydes are omnipresent as components of the odor of green leaves (Visser et al., 1979). Thus one could predict the presence of many receptors on the antennae of the worker ants since green plants and associated green odors form a large part of their environment via the mound construction. Mean response of the brood tenders to the standard was −0.38 mV (SE = 0.04; N = 3). Each stimulus was either preceded or followed at 4 min by a stimulation with the standard. Responses to intervening test stimuli were represented as a percent of the mean of the two nearest responses to the standard (Dickens, 1978, 1981). The size of the EAG was considered to be a measure of the relative number of responding acceptors (Payne, 1975; Dickens and Payne, 1977).

RESULTS AND DISCUSSION

The results of the bioassays are given in Tables 1–4. In Y-tube olfactometer trials with intact, live larvae, it was determined that 300 immatures were capable of eliciting a statistically significant response from brood tenders (Table 1). Worker response to sibling larvae was not significantly different from worker response to heterocolonial, field-collected brood. These data strongly suggest that worker response to a brood pheromone attractant does not require contact chemoreception and that this response is not mediated by worker conditioning to colony-specific brood odors, be they heritable or environmental. Similar evidence for the presence of a volatile attractant given off by brood of the RIFA is presented in the work by Lofgren et al. (1983). In olfactometer bioassays in which ants were given the option of orienting to an airstream blown over their own colony queen or to an airstream being blown over larvae from their own colony or from an alien colony, the ants responded equally to the airstream over their own larvae and over alien larvae. The implication here is

TABLE 1. OLFACTOMETER RESPONSE OF BROOD TENDERS TO 300 LIVE, INTACT IMMATURES[a]

Response to	No. attracted ($X \pm$ SD)	No. not attracted ($X \pm$ SD)	χ^2 (1 df)
Own brood	161 (26.83 ± 2.32)	79 (13.17 ± 2.32)	10.44
Alien brood	159 (26.50 ± 2.74)	81 (13.5 ± 2.74)	9.57

[a] Laboratory-reared workers were tested against brood from their own colony and against field-collected brood; N = 6.

that regardless of the source of the brood, or the brood–worker kinship, a volatile is present that causes attraction. Observations in the field have shown that when a colony is disturbed and some of the larvae are buried beneath the soil, the worker ants are quite adept at locating each and every piece of brood. It is quite possible that the workers are locating the buried brood by the presence of the brood pheromone. It might be argued that the workers are using substrate vibrations produced by the larvae to locate them. However, it has not been shown that RIFA larvae are capable of producing sound. Lenoir (1984) has shown that, in research with the ant *Cataglyphis cursor*, colony workers recognize sibling brood immediately. If, however, workers have been adopted into the colony, these adoptees do not initially recognize the larvae as sisters. However, after six days, the adopted workers begin to tend brood indicating the inhibition has disappeared. Lenoir's work not only demonstrates the presence of a brood-tending pheromone, but also presents data that show brood kin recognition.

Further evidence for the perception by brood tenders of odorous stimuli emanating from the larvae was obtained from the electrophysiological studies. A dose–response curve constructed from EAGs to increasing larval equivalents showed receptor response to increase from a threshold of ca. 1–10 larval equivalents to a saturation level of ca. 10–100 larval equivalents (Figure 1). As seen in Figure 1, the maximum response to the brood rinse was 20% of the 1-hexanol

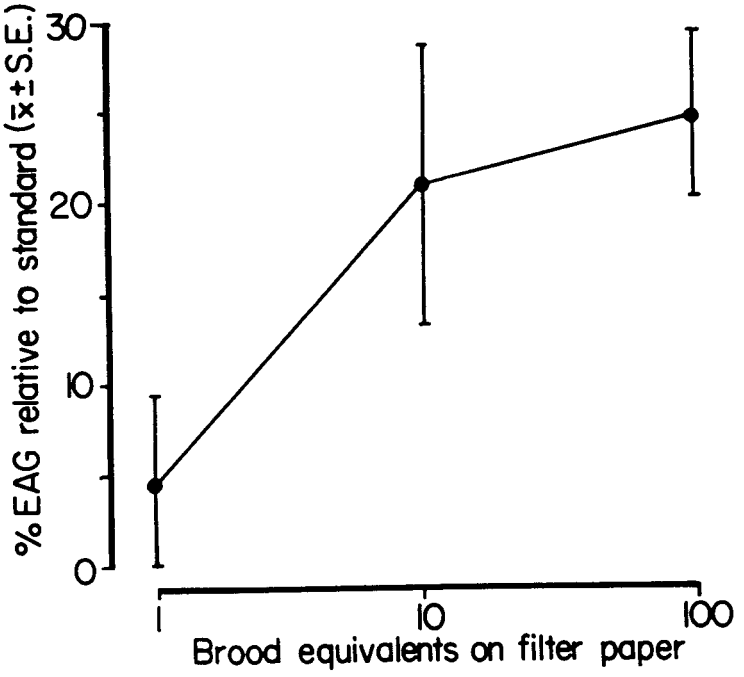

FIG. 1. Dose–response curve constructed from mean EAGs of brood tenders ($N = 3$) to increasing brood equivalents on filter paper. Vertical bars represent standard errors.

TABLE 2. RESPONSE OF BROOD TENDERS FED TWO DIFFERENT DIETS IN SPOT ATTRACTION BIOASSAY

Treatment	Response, % ($X \pm$ SE)	
	Normal diet	Roach diet
Brood rinse	79 ± 2.3A[a]	57.8 ± 5.7G
Larval resting spot	14 ± 2.4B	18.6 ± 4.2H
Roach rinse	4 ± 1.2C	3.0 ± 1.1I
Solvent	3 ± .75C	1.6 ± 0.6I

[a] Letters following SE in each column refer to within column comparisons (Duncan's new multiple-range test) where means followed by different letters are significantly different ($P < 0.0001$).

standard. One may wonder if this is indeed a significant response and why brood tenders should exhibit greater EAG responses to the leaf volatiles than to the brood pheromone. The green leaf volatiles may be detected by numerous receptors with broad specificity, while the brood pheromone receptors may be few in number but highly specific. A situation similar to the one described above occurs in the boll weevil (Dickens, 1984, and unpublished data). Furthermore, disregarding the peripheral receptor system, higher order neural processing may amplify and modulate plant odors and pheromones quite differently. Thus, in this case, the 20% response does indeed represent a significant value. Little or no response was obtained with the volatiles from triolein.

The results from the brood rinse experiments (Tables 2–4) show that some kind of attractive material is being extracted from the larvae. In the spot attraction test, regardless of the type of diet, the workers responded best to the rinse of the larvae (Table 1). In this test, where workers have a choice of orienting to a particular spot and settling down, the implication is that there is a contact

TABLE 3. RESPONSE OF BROOD TENDERS FED TWO DIFFERENT DIETS IN SURROGATE BIOASSAY

Treatment	Response, % ($X \pm$ SE)	
	Normal diet	Roach diet
Brood rinse	14.4 ± 3.30A[a]	20.3 ± 2.8G
Roach rinse	1.8 ± 0.56B	0.0 ± 0.0H
Solvent	1.6 ± 0.55B	2.6 ± 0.8I

[a] Letters following SE in each column refer to within column comparisons (Duncan's new multiple-range test) where means followed by different letters are significantly different ($P < 0.0001$).

TABLE 4. RESPONSE OF RIFA WORKERS FROM DISRUPTED LABORATORY COLONIES TO PIECES OF CONSTRUCTION PAPER IMPREGNATED WITH VARIOUS RINSES

Treatment	Average retrieval to colony or colony area[a]	
	Normal diet	Roach diet
Brood rinse	83	80
Roach rinse	0	0
Solvent	0	0

[a] Mean response based upon 10 pieces of paper for each rinse presented to five separate colonies.

material present that causes the workers to respond. In the natural setting of a colony, the brood tenders do not wander about, but tend to remain on the brood pile while carrying on their activities. This tendency to settle down is seen also in the response to the spot upon which the larvae had rested. These responses to the larval rinse spot or the larval resting spot agree with the findings of Walsh and Tschinkel (1974) that a pheromone is present that is intimately tied up with the cuticle. However, their conclusion was that the workers needed to contact the brood in order for a response to follow. Our data show that this latter conclusion may not necessarily be true.

Cockroach extracts were not active in tests with disrupted colonies. Ants from both tests responded to small pieces of paper treated with the larval rinse by stacking scattered brood upon the papers, by building trails from the nest to the papers, and by returning the papers to the area of the nest. No papers treated with the cockroach rinse or the solvent control were treated in this manner.

Data from both behavioral bioassays and electrophysiological studies show that volatile compounds are associated with RIFA larvae and that these compounds are capable of eliciting a response by brood tending workers. The failure of Walsh and Tschinkel (1974) to detect a volatile compound might be explained by the use of workers other than brood tenders and the extremely small amounts of the pheromone present on single larvae. We found it necessary to use at least 300 larvae to elicit an olfactometer response. Response by the workers to brood cuticle suggests that the brood recognition pheromone may have at least two components: one, a volatile component that draws the workers near the brood; and two, a contact material that causes retrieval behavior. The two components together evidently have a very shallow active space around each larva.

The question arises as to whether the pheromone is produced by the larvae or whether it is the result of some material being applied to the larvae. We know that when the RIFA queen lays an egg, she draws the ovipositor over the

egg and applies venom to it (VanderMeer, unpublished data). This material may serve not only as an antibiotic, but also as a means to alert the brood tenders to carry the egg to the brood pile. An egg-laying queen has an entourage of workers that mill about her posterior end during the egg-laying period. When an egg is deposited and the sting drawn across it, these workers enter a very highly excited state. The egg is quickly picked up and taken to the brood pile. Another possibility for the origin of the material may be the mere physical transfer of worker hydrocarbons to the brood. These hydrocarbons may facilitate the brood tender's feeding and grooming of the larvae. Such behavior is already known in the case of the myrmecophilous beetle, *Myrmecaphodius excavaticollis* (VanderMeer and Wojcik, 1982). This RIFA symbiont is capable of acquiring the RIFA hydrocarbon pattern and, being so marked, is able to move freely about the colony and obtain food directly from workers. Finally, the attention paid to the brood may simply be a result of the topical application of venom by the brood tenders. Obin and VanderMeer (1985) have shown that worker ants disperse venom through the air by raising the abdomen and vibrating the gaster (termed "gaster flagging"). Although these authors suggest that this behavior is a method of dispersing antibiotics, it may also be that it is the method of marking the brood for tending behavior. Regardless of the source of the material that causes brood tending, it is obvious that some chemical is present and that this material is capable of being extracted and tested.

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