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Source of Alate Excitant Pheromones in the Red Imported Fire Ant *Solenopsis invicta* (Hymenoptera: Formicidae)

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At the onset of mating flights in Solenopsis invicta, *workers swarm excitedly over the mound as alates prepare to fly. Previous studies demonstrated that this excitement is stimulated by the male and female alates. We investigated the glandular source(s) of pheromones produced by the alates that cause excitement. The only common female and male alate body part that elicited excitement when crushed was the head. Within the head, excised mandibular glands were found to be responsible for worker excitement. Fire ant workers are very sensitive to external stimuli and some excitement was elicited by crushed female gasters and male thoraces, but the response was never as significant as with crushed heads. Tests with summer and winter alates revealed similar results, except that gasters of winter female alates had a greater excitant effect than did gasters of summer female alates. This may be due to the production of attractant pheromones by the poison glands of overwintering female alates. We conclude that the mandibular gland is the source of alate excitant pheromones.*

KEY WORDS: *Solenopsis invicta*; alates; excitant pheromones; mandibular glands; alarm.

INTRODUCTION

Reproduction in the red imported fire ant, *Solenopsis invicta* Buren, begins with synchronized nuptial flights during which male and female alates (winged reproductives) leave their natal nests and mate several hundred meters in the air (Green, 1952; Markin *et al.*, 1971). Males typically take flight before females and form large aggregations into which the females fly (Markin *et al.*, 1971).

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Mating flights may take place year-round but occur primarily during the summer months, when suitable conditions most frequently arise (Bass and Hays, 1979). Alates of *S. invicta* usually fly shortly after a rainstorm, when soil temperatures are between 24 and 32°C and surface wind speeds are low (Rhoades and Davis, 1967; Morrill, 1974; Milio *et al.*, 1988).

The surface of a fire ant mound normally has no entrance or exit holes, as workers traverse their territory via underground foraging tunnels that have exit holes to the surface (Markin *et al.*, 1975). However, just prior to a mating flight, workers create holes in the mound surface from which workers and winged alates emerge (Markin *et al.*, 1971). At this time, workers swarm excitedly over the mound and exhibit many characteristics of what has been classified as alarm behavior, including frenzied running, rapid back-and-forth movement, and increased aggression (Markin *et al.*, 1971; Obin and Vander Meer, 1994). In addition, workers often aggregate around alates as they climb up vegetation to take flight and sometimes attempt to pull the alates back down.

Heightened worker excitement and aggression at the onset of mating flights has been observed in several other ant species, including leaf cutting ants, *Acromyrmex* spp. and *Atta* spp. (Fowler, 1982), and several species of *Pogonomyrmex* (Hölldobler, 1976). The purpose of this heightened worker activity may be to protect and aid alates as they prepare to fly or to ready workers for attacks against newly mated queens attempting to initiate colonies near their mounds (Fowler, 1982).

The excitement exhibited by *S. invicta* workers at the time of mating flights is similar to that typically classified as alarm behavior but is not in response to a disturbance or potential danger, which are the usual conditions under which alarm pheromones are released (Parry and Morgan, 1979; Hölldobler and Wilson, 1990). Alarm behaviors may include frenzied erratic movement, raised heads, and outstretched antennae (Duffield *et al.*, 1977), attraction, increased speed, and orientation toward the source (Cammaerts *et al.*, 1985), and spread mandibles, biting, and spraying of poison gland products (Blum *et al.*, 1988). Wilson and Regnier (1971) divided alarm behavior into two categories: "panic alarm," characterized by rapid excited bursts of nondirectional movement, and "aggressive alarm," in which workers run excitedly toward the source of the alarm substance, assume a defensive posture, and often attack alien objects at the source of the disturbance.

Obin and Vander Meer (1994) induced *S. invicta* flights in the laboratory and showed that chemical cues from both male and female alates, but not from workers, attracted workers, induced alarm-recruitment behaviors in the workers, and promoted alate retrieval by workers. They proposed that volatile substances produced by the alates were responsible for eliciting the worker reactions. In

this paper, we investigated the glandular source(s) of chemical substances responsible for inducing worker excitement in *S. invicta* workers prior to mating flights.

METHODS

Sources of *S. invicta* Alates

Queen-right, monogyne *S. invicta* colonies containing alates ($n = 24$) were collected from field sites near Gainesville, FL, and set up in their own soil in large rubber utility tubs in the laboratory (Rubbermaid Co., 16-gal Roughneck tubs, 55.9 × 54.6 × 36.8 cm). Colonies were maintained at room temperature ($\approx 23°C$) and provided with water presented in a large test tube plugged with cotton, a similar test tube with 10% sucrose solution, and frozen crickets (three times a week). Tongue depressors were inserted into the soil to act as launching sites for mating flights.

Colonies were induced to fly in the laboratory by watering the soil of each colony 1 day prior to testing to simulate rainfall. On the day of each test we increased the temperature in the lab room to 30°C and augmented the available light with incandescent lamps. A soil temperature $\geq 24°C$ (see Markin *et al.*, 1971) and sufficient light were necessary for flight initiation.

"Mating flight-activated" (MFA) alates were obtained from these laboratory colonies once a flight was initiated. Alates were collected as they climbed up tongue depressors placed in the tubs. Alates were weighed and tested immediately after collection.

"Nonflying" alates were collected directly from monogyne *S. invicta* colonies at field sites near Gainesville, FL. Alates were housed in petri dishes with wet cotton and a few workers from their colony for less than 2 days prior to use. All alates were weighed before testing.

Schedule of Testing

MFA female alates from colonies collected in the winter months of 1995 (off season for mating flights, February–April) and in the summer months of 1995 (high season, June–August) were tested separately to investigate seasonal differences in pheromone production. Body parts and glands from MFA female alates were also tested in both flight seasons. Live nonflying female alates were tested in both seasons but body parts were tested in the winter only. MFA male alates, body parts, and glands were tested in the summer of 1995, while nonflying males and body parts were tested in the winter of 1996.

Bioassay Procedure

Our bioassay consisted of worker groups from each of 13–17 mature *S. invicta* colonies maintained in the laboratory. Workers and test alates were always collected from different colonies. Approximately 100 workers and a small amount of brood from each colony were placed in small covered petri dishes (5.3 cm in diameter × 0.5 cm deep) with moist Castone (Dentsply Co.) bottoms. Red cellophane placed over the petri dishes induced the workers to stay with their brood in the dishes. These subcolonies were maintained in individual plastic trays (22.5 × 7.5 × 5 cm) that were coated with Fluon (ICI Fluoropolymers Co.) on the sides to prevent escape. Ants were allowed to acclimate to the dishes for at least 2 h before the lids and cellophane were removed. Ants were left for 2 additional h before testing began to ensure that they were calm for testing. Sometimes the ants aggregated between the dish and the side of the tray. As long as the workers were calm, samples were tested on the groups wherever they clumped.

The bioassay was conducted by an observer and assistant. The assistant prepared test samples for the observer so that the observer did not know the sample identity. Test samples consisted of 3 ml of air drawn by a syringe (5 ml, plastic; Henke-Sass Wolf Co.) from a control or sample vial. The assistant then assigned each sample arbitrarily to a worker group, such that all worker groups were tested with each of the test samples and controls. The observer positioned the syringe 1–2 cm directly above a part of the selected worker group, then slowly released 1 ml of air over the workers. Reactions were characterized by the observer either as no reaction, when workers did not change their behavior in any way or simply raised their heads and antennated the air, or as an excited reaction, in which at least one worker reacted with rapid movement. If workers moved rapidly toward the source of the airstream, the reaction was noted as possible attraction. All tests were conducted between 1300 and 1600 EST at a room temperature of 25–28°C and with standard overhead fluorescent lighting.

Three to six samples were tested in each bioassay, with at least 5 min passing before the same worker group was retested. The order of testing was recorded to check that none of the test samples had a prolonged effect on the behavior of any worker groups. Air from an empty vial was tested as a negative control and 5 female alates (shaken) or 10 workers (shaken) were used as a positive control. Five microliters of mineral oil on filter paper was used as the control in tests of glandular solutions. Only those test runs in which appropriate reactions to the negative and positive controls were obtained were used for analysis. Each test sample was assayed at least three times, with each replicate utilizing a new set of worker groups.

Sample Preparation

Each sample to be tested in the bioassay was placed in a 7-ml glass scintillation vial and tightly capped. Tests with live alates consisted of five live alates placed in a vial and shaken immediately before each air sample was drawn. Vials were shaken in order to disturb the alates and induce them to release the excitant pheromone. Five live female alates that were not shaken (calm) were tested to investigate whether alates were able to control release of the pheromone. Tests of alate body parts included one individual head, thorax, or gaster. Each body part was obtained from different alates to minimize cross contamination. Alates were chilled to 8°C before body parts were separated using micro dissecting scissors. Each body part was placed toward one end of a thin strip of filter paper (Whatman No. 1, qualitative, 3 × 0.5 cm), then the filter paper was folded over and the body part was crushed with a hammer. The filter paper with the crushed body part was immediately placed in a vial for bioassay.

Solutions of glandular products were made from mandibular glands, postpharyngeal glands, and poison sacs excised in water under a binocular dissecting microscope from alates that had initiated preflight activity. Each solution consisted of 20 mandibular glands (with mandible attached), 10 postpharyngeal glands, or, from females only, 10 poison sacs (including the poison gland, reservoir and Dufour's gland) macerated in 30 μl of mineral oil. Mineral oil was used to slow the rate of release of the chemical compounds. For each test, 5 μl of solution (1.6 alate equivalent) was applied to a thin strip of filter paper (dimensions above) and placed in a vial. Solutions were refrigerated until use.

Attraction Bioassay

A Y-tube olfactometer was used to investigate whether poison sacs were attractive to workers (see Methods in Vander Meer *et al.*, 1988). Poison sac solutions, each consisting of 10 poison sacs macerated in 300 μl hexane, were prepared from winter MFA female alates and summer MFA female alates. Ten microliters of solution was used in each test (0.3 alate equivalent).

Data Analysis

Data were analyzed using the McNemar test for significance of changes (Sokal and Rohlf, 1981). This statistic compares the number of worker groups that displayed an excited reaction to the test sample but not to the negative control to the number of groups that reacted to the negative control but not to the test sample. Because this statistic produces a modified *G*-value, data from the three to nine replicates per test sample could be combined to obtain an overall pooled *G*-value. If the total of the individual replicate *G*-values was not

significantly different from the pooled *G*-value, then the results were considered homogeneous [heterogeneity *G*-test (Sokal and Rohlf, 1981)] and the pooled *G*-value was used for comparisons between test samples. Replicates were heterogeneous if the number of worker groups reacting to the test samples was not always greater than the number reacting to the control or if the magnitude of this difference was variable. In these cases, results from individual replicates were considered and the percentage of significant replicates was calculated.

RESULTS

In response to air from vials containing live shaken alates, *S. invicta* workers displayed heightened excitement which consisted of the rapid erratic movement typical of "panic alarm." Live MFA female alates elicited highly significant excited reactions in the worker groups in both the summer (Fig. 1) and the winter (Fig. 2) tests. Live winter MFA female alates that were not shaken before testing also elicited significant reactions (Fig. 2). In addition, workers reacted with excitement to summer MFA male alates (Fig. 1) as well as to winter nonflying female and male alates (Fig. 3).

Colonies collected during the winter months usually flew between 1200

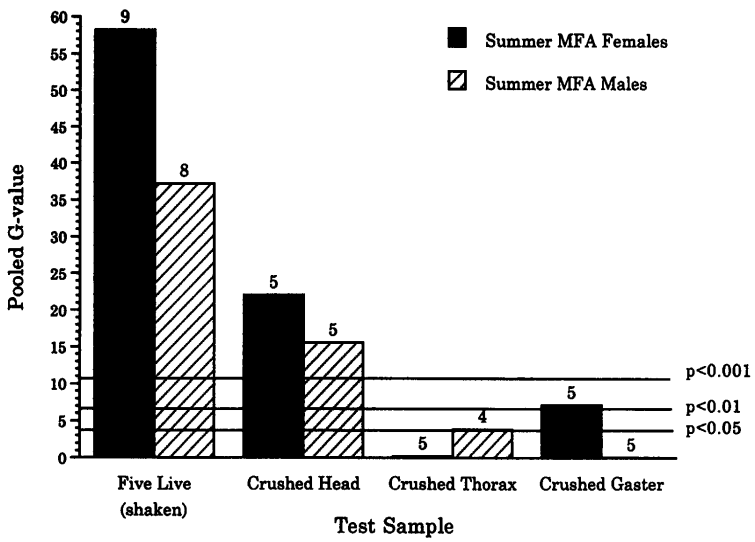

Fig. 1. Pooled *G*-values from replicated bioassays testing excited reactions in workers of *Solenopsis invicta* to female and male alates that initiated flight (MFA alates) in the laboratory during the summer months. Data were analyzed using the McNemar test for significance of changes. Levels of significance (*P* values) are shown for df = 1 and the number of replicates for each sample is indicated above the bars.

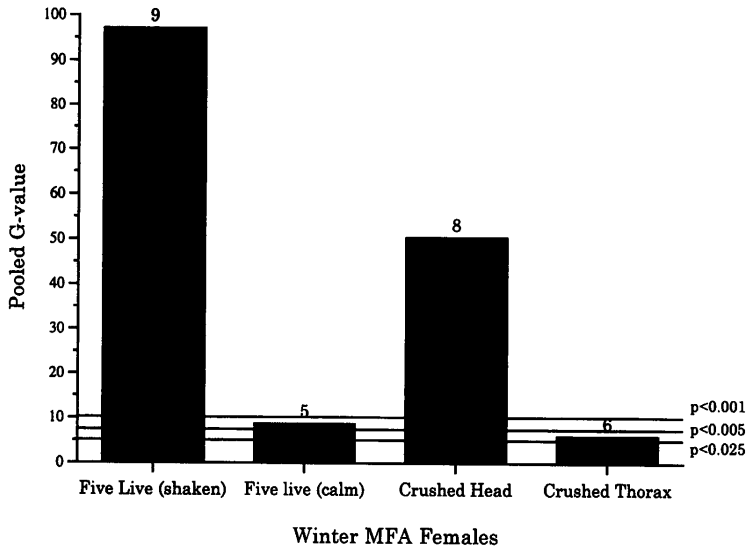

Fig. 2. Pooled *G*-values from replicated bioassays testing excited reactions in workers of *Solenopsis invicta* to female alates that initiated flight in the laboratory during the winter months. Data were analyzed using the McNemar test for significance of changes. Levels of significance (*P*-values) are shown for df = 1 and the number of replicates for each sample is indicated above the bars. Results for crushed gasters are not shown because *G*-values were heterogeneous and could not be pooled (see Results).

and 1500 EST, while colonies collected in the summer flew between 1100 and 1300 EST. Winter MFA female alates weighed significantly less than summer MFA female alates [mean winter weight, 10.53 ± 0.22 mg (±SE), $n = 50$; mean summer weight, 13.33 ± 0.22 mg, $n = 39$; Student's t test, $t = -8.93$, df = 87, $P < 0.001$). The mean weight of winter nonflying female alates (11.14 ± 0.18 mg; $n = 25$) did not differ from that of winter MFA female alates ($t = -1.828$, df = 73, $P = 0.07$). The mean weight of summer MFA males (6.84 ± 0.07 mg; $n = 47$) did not differ from that of winter nonflying males (6.89 ± 0.11 mg; $n = 47$; $t = -0.35$, df = 91, $P = 0.73$).

Crushed heads of all alate categories stimulated highly significant excitement in the workers (Figs. 1–3). Results for crushed female thoraces were variable, while crushed male thoraces elicited excitement at low levels of significance (Figs. 1–3). Crushed female gasters but not male gasters elicited excitement (Figs. 1 and 3). Tests with winter MFA females were heterogeneous; seven of the eight replicates had trends in the same direction, but only four (50%) of these trends were significantly different from the control.

Mandibular gland solutions from summer MFA female and male alates produced significant excitement in workers (Table I). Tests with winter MFA

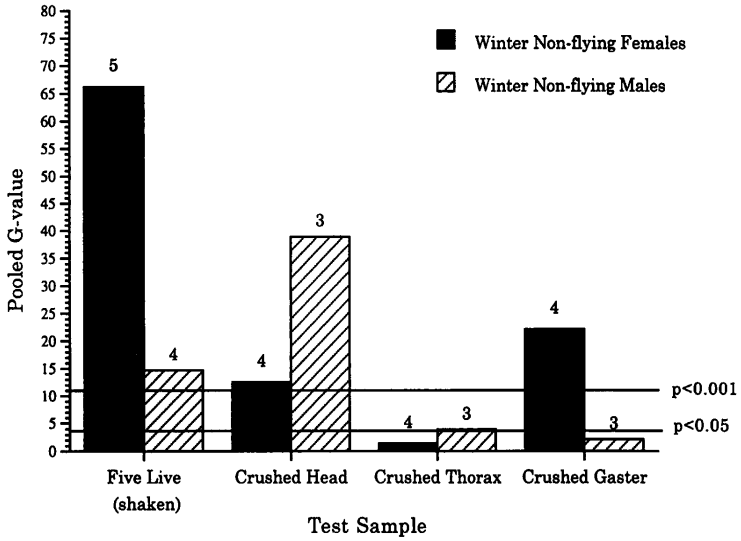

Fig. 3. Pooled *G*-values from replicated bioassays testing excited reactions in workers of *Solenopsis invicta* to nonflying female and male alates collected from colonies during the winter months. Data were analyzed using the McNemar test for significance of changes. Levels of significance (P values) are shown for df = 1 and the number of replicates for each sample is indicated above the bars.

females were heterogeneous, with four highly significant replicates (80%; each with $P < 0.005$) and one with an equal number of reactions to the test sample as to the control. Crushed heads without mandibular glands did not elicit excitement in workers (Table I; $P > 0.05$), while heads with intact glands tested at the same time elicited excitement at a highly significant level ($P < 0.001$).

No excitement was elicited by postpharyngeal gland solutions from any of the alate categories (Table I). Only 1 of 15 tests was significant. Worker reactions to poison sac solutions from MFA female alates differed between flight seasons. While solutions from summer alates did not elicit a significant response, 60% of the replicates with winter alates were significant (Table I). Heterogeneity in the winter alate tests was due to three highly significant replicates (each with $P < 0.001$), one replicate with a similar but nonsignificant trend, and one replicate in which the glandular solution did not differ from the control.

In the Y-tube bioassay, workers from laboratory colonies were attracted to the poison sac solution made from winter MFA female alates (*G*-test of goodness of fit, $G = 6.09$, df = 1, $P = 0.001$) but not to the poison sac solution from summer MFA alates ($G = 0.27$, df = 1, $P = 0.61$).

Table I. Results of Bioassays Testing Excited Reactions in Workers of *Solenopsis invicta* to Solutions of Glands Excised from Female and Male Mating Flight-Activated (MFA) Alates[a]

Category	No. replicates	Homogeneous		Heterogeneous (% of replicates significant)
		Pooled *G*-value	*P* value (df = 1)	
Mandibular gland solution				
Winter females	5	—	—	80
Summer females	5	6.04	<0.025	
Summer males	5	6.34	<0.025	
Postpharyngeal gland solution				
Winter females	5	—	—	20
Summer females	5	—	—	0
Summer males	5	0.33	>0.05 (NS)	
Poison sac solution				
Winter females	5	—	—	60
Summer females	5	1.99	>0.05 (NS)	
One crushed female head				
Without mandibular glands	3	2.14	>0.05 (NS)	
With mandibular glands	3	16.14	<0.001	

[a]Data were analyzed using the McNemar test for significance of changes. Pooled *G*-values were calculated when results from replicates were homogeneous (see Methods). The percentage of replicates that was significant is reported for categories in which replicates were heterogeneous.

DISCUSSION

Our results show that the mandibular glands are a source of an excitant pheromone in both female and male *S. invicta* alates. In our bioassay, *S. invicta* workers consistently reacted with rapid movement and frantic running when exposed to live alates, crushed heads, and mandibular gland solutions (Figs. 1–3). These results support Obin and Vander Meer's suggestion (1994) that the "alarm" and recruitment reactions exhibited by workers toward alate residues during mating flights were likely derived from the alate mandibular glands.

Although other crushed body parts from male and female alates elicited excitement, only the head from both sexes of alates elicited a strong reaction. This is significant because within a fire ant population, colonies may produce only males, only females, or both sexes. It is likely the alates that are responsible for initiating mating flight activity in response to environmental conditions, because the opening of the mound surface and swarming of workers are associated only with mating flights. It is also probable that the glandular source for this very specific activity is the same for both sexes. Thus, all evidence points to the mandibular glands as the source of mating flight excitant pheromones.

We found that excitant substances were released by both MFA and non-flying alates when shaken, indicating that alates are able to release these substances in contexts other than mating flights. This was also observed by Obin and Vander Meer (1994), as workers were attracted to and excited by vials containing residues of alates that had not been involved in a mating flight. If similar excited reactions are elicited in response to a disturbance or perceived danger to the colony, then these excitant substances may also function as true alarm pheromones. This is supported by results from tests with live MFA female alates that were not shaken in the vial. These samples elicited worker excitement, indicating that the action of collection, transport, and placement of the alates in the test vial triggered the release of excitant (alarm) pheromones. It is unlikely that the alates are always releasing small amounts of the excitant pheromone since, if that were true, some workers would be excited at all times.

The excited response to mandibular gland solutions was less pronounced than that elicited by live alates or by crushed heads. This was probably due to the volatile nature of the compounds involved. These excitant pheromones are likely similar in volatility to alarm pheromones, which are known to be comprised of small molecules that disperse rapidly when released (Wilson and Bossert, 1963; Blum, 1969). Because attempts to use glands crushed on filter paper were unsuccessful, glands were crushed in mineral oil in an attempt to slow the release of the compounds. Clearly, active glands in live alates, where the excitant compounds can be continually produced and released, cannot be directly compared to excised glands. It is most probable that under the conditions of dissection and sample preparation, biosynthesis of the compounds is halted once the gland is excised and loss of volatile compounds during the dissections and sample preparation is unavoidable.

The excitement displayed by *S. invicta* workers consisted of typical "panic alarm" behaviors (Wilson and Regnier, 1971). We observed few instances of attraction to the test samples (observed primarily with crushed female gasters and poison glands; see below) and no aggressive behaviors such as mandible gaping or aggressive posturing. Our bioassay did not allow the workers to contact the source of the substance, which may be required to provoke an aggressive response. When ants exhibit an aggressive alarm reaction they usually move toward the source of a disturbance, where they attack alien objects (Wilson and Regnier, 1971). Most bioassays of other ant species that have shown aggressive reactions to alarm pheromones were conducted with test substances applied on alien material that could be contacted by the workers (e.g., Tomalski *et al.*, 1987; Kugler, 1979; Hölldobler *et al.*, 1990). Bioassays that prohibited contact between the ants and a physical object usually resulted in rapid panic alarm or attraction to the source but no defensive posture or attacking (e.g., Wilson, 1958; Duffield *et al.*, 1977; Blum and Amante, 1981; but see Blum *et al.*, 1968). Tricot *et al.* (1972) proposed that the alarm pheromones of *Myrmica*

rubra may be comprised of inhibitory and stimulative components that direct the aggressiveness of alarmed workers toward the cause of the disturbance instead of toward the worker emitting the alarm pheromone.

Crushed gasters of all female alate categories produced some level of excitement in the workers, as did crushed poison gland solutions from winter MFA females. These results might be explained by considering the effects of overwintering on female alates. Female alates that are unable to fly during the summer flight season overwinter in their natal nest until conditions are appropriate for a mating flight. Overwintering female alates are known to be slightly abnormal in that they lay eggs in the presence of their queen (Fletcher and Blum, 1983) and produce a queen attraction pheromone which is usually only released by inseminated queens or virgin females that have shed their wings (Glancey *et al.*, 1981). This queen pheromone is produced by the poison gland and is highly attractive to workers (Vander Meer *et al.*, 1980). Jouvenez *et al.* (1974) found that virgin alates could also be slightly attractive to workers.

If the winter female alates in this study were producing queen pheromone, then the reaction elicited by the gasters or poison sacs of winter females in our excitement bioassay may have been confounded by attraction of the workers to the queen pheromone. The results from our Y-tube attraction bioassay support this hypothesis, since we found that poison sacs from winter MFA alates but not summer MFA alates were attractive to workers. However, this does not explain the diminished, yet significant, result for summer female alates, whose poison sac did not cause an excited reaction. There appear to be volatile compounds from the female gaster that trigger excitement in the sensitive fire ant workers.

Thoraces from males and winter MFA females caused low levels of excitement. It is not clear yet whether this is due to contamination by substances released from the mandibular glands during separation of the body parts or if a glandular source from the thorax is responsible.

That there may be multiple sources of excitant pheromones in *S. invicta* is not surprising. Alarm communication in most ant species has been shown to involve a complex pattern of behaviors elicited by a combination of chemical compounds (Bradshaw *et al.*, 1975, 1979; Wheeler *et al.*, 1975; Cammaerts *et al.*, 1983). In addition to blends of pheromones from a single exocrine gland, chemical signals can be derived from multiple glandular sources (Blum, 1969; Hölldobler, 1995). Robertson (1971) found that alarm behavior in *Myrmecia gulosa* is controlled by a number of chemicals from the mandibular and rectal glands that each elicit different parts of the alarm reaction. Several other studies have also found evidence of multiglandular sources of alarm pheromones (Tricot *et al.*, 1972, Cammaerts-Tricot, 1973) and recruitment pheromones (Hölldobler and Wilson, 1970; Attygalle and Morgan, 1985).

Studies of chemical communication associated with mating flights have

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