



# The Role of Chemical Senses in Seed-carrying Behavior by Ants: A Behavioral, Physiological, and Morphological Study

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The sensory bases of seed-carrying by ants were studied through behavioral, physiological, and morphological methods. Fourteen colonies from 4 ant species [*Aphaenogaster rudis* Emery, *Lasius alienus* Foerster, *Formica subsericea* Say, and *Camponotus ferrugineus* (F.)] were behaviorally tested for olfactory and gustatory responses to seeds of *A. canadense*. Results indicate that ants do not perceive these seeds, which have elaiosomes, by olfaction. Rather, they respond to these seeds only if the seeds are antennated. These results indicate that these ants are not “attracted” to *A. canadense* seeds by olfactory cues. Electroantennograms (EAGs) recorded from *F. subsericea* in response to olfactory and gustatory application of mixed isomer diolein, a major component of many seed elaiosomes, support the behavioral data. Olfactory delivery produced EAG responses no different from that of controls, while gustatory delivery produced responses with dose-dependence. Scanning electron microscopy (SEM) of the antennae of *A. rudis*, *F. subsericea*, and *C. ferrugineus* revealed the presence of sensilla chaetica, presumed to be contact chemoreceptors, and sensilla placodea and/or sensilla trichodea curvata that are believed to be olfactory receptors.

Ants Myrmecochory Seed-carrying behavior EAG Chemical senses

## INTRODUCTION

Seed dispersal by ants occurs in over 80 plant species, by ants in 4 of the 7 ant subfamilies (Beattie, 1983). The ant-dispersed plants, the myrmecochores, produce seeds having conspicuous food bodies called elaiosomes. Elaiosomes contain proteins, sugars, and lipids (Bresinsky, 1963 cited in Brew *et al.*, 1989; Skidmore and Heithaus, 1988; Brew *et al.*, 1989; Lanza, *et al.* 1992) that are used by ants for food (Beattie, 1983). Ants typically carry these diaspores (seeds + elaiosomes) to their nests and either eat the elaiosomes themselves, or feed the elaiosomes to their larvae, discarding the seeds onto waste middens (Davidson and Morton, 1981; Horvitz, 1981; Beattie, 1983). Seeds treated in this manner may benefit in several ways: (1) by escape from predators (Culver and Beattie, 1978; Westoby *et al.*, 1991) and/or competitors (Gunther and Lanza, 1989) and/or (2) by nest chemistry that promotes the vigor of the seedling (Beattie

and Culver, 1983; Oostermeijer, 1989; Levey and Byrne, 1993). The latter point has been disputed by a number of studies showing that in some systems, the soil chemistry around ant nests is not different from non-nest areas (Rice and Westoby, 1986; Westoby *et al.*, 1991), or that seedling growth is not increased in nest areas despite an increase in nitrates and other nutrients (Horvitz and Schemske, 1986a). Alternatively, the removal of the elaiosome and seed coat from the diaspore may enhance germination by scarifying the seed coat (Culver and Beattie, 1980; Horvitz, 1981). There is also evidence that the elaiosome itself is repellent to deer mice, preventing predation of the seed (Hanzawa *et al.*, 1985).

We must also note that the carrying of seeds by ants is variable depending on the ant species, the plant species, and the context in which the interactions occur. For a given seed, its removal depends on which ant species are present, as some are more likely to transport certain seeds than others (Bossard, 1981; Davidson and Morton, 1981; Kjellsson, 1985; Horvitz and Schemske, 1986b; Mossop, 1989; Oostermeijer, 1989). The removal of a given seed also depends on the presence of other types of seeds and their density, for ants will express a preference for certain seeds over other seeds (Oostermeijer,

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1989). Last, the probability of a seed being moved is context-dependent, for both distance from the nest (Pemberton, 1988) and the patchiness of the nests (Smith *et al.*, 1989) have been shown to influence the probability of seed removal.

The benefits of myrmecochory to both plants and ants are quite well-established, while the behavioral bases of seed-dispersal by ants has remained less clear. Several hypotheses have been put forth to explain the ants' behavior. The elaiosome/diaspore mass ratio hypothesis (Gunther and Lanza, 1989) has been proposed to explain the variability in seed-carrying by ants. Under this hypothesis, ants preferentially remove diaspores with heavier elaiosomes because of the greater food reward. Both in cases where seeds had natural variations in elaiosome size (Oostermeijer, 1989; Gunther and Lanza, 1989) and where elaiosome/diaspore mass ratios were manipulated (Hughes and Westoby, 1992), it was found that seeds with the larger elaiosomes, or higher elaiosome/diaspore mass ratios were preferred by ants. In other studies where ants were given the choice of seeds without elaiosomes, intact seeds with elaiosomes and/or elaiosomes alone, ants typically removed few of the seeds lacking elaiosomes while preferentially removing either the intact seeds or the elaiosomes alone (O'Dowd and Hay, 1980; Kjellsson, 1985; Hanzawa *et al.*, 1985; Pemberton, 1988; Oostermeijer, 1989; Brew *et al.*, 1989). Some ants have also been observed removing the elaiosome from the seed and then carrying only the elaiosome back to the nest (O'Dowd and Hay, 1980; Bossard, 1991).

Three other hypotheses have been put forth to explain why ants carry seeds. The general nutrient content hypothesis (Brew *et al.*, 1989) proposes that ants remove seeds for their general nutrient content. If so, in experimental conditions where elaiosome fractions are applied on inert piths and presented separately to ants, ants should collect the most abundant chemical fractions rather than the less abundant fractions. Alternatively, the ants may collect all elaiosomes fractions equally (Brew *et al.*, 1989). This appears not to be the case as ants preferentially removed piths with minor components of the elaiosomes (Brew *et al.*, 1989).

The second hypothesis is the essential nutrient content hypothesis (Brew *et al.*, 1989), in which ants remove the seeds because they contain a nutrient essential to ant function. The evidence, however, does not support this hypothesis as many, if not all, of the elaiosome components can be synthesized by ants (Brew *et al.*, 1989).

More recently, the chemical behavior-releaser hypothesis has received support (Skidmore and Heithaus, 1988; Brew *et al.*, 1989; Kusmenoglu *et al.*, 1989; Gunther and Lanza, 1989; Lanza *et al.*, 1992). In this hypothesis, one or more chemical components of the seed or elaiosome induces the seed-carrying behavior from the ants. Evidence of such chemical signals is well-known among ants: oleic acid, for instance, has been shown to be a stimulant for corpse-carrying behavior in *Solenopsis*

(Wilson, 1971) and in *Pogonomyrmex* (Gordon, 1983). In the latter study, the effect of oleic acid was found to be heavily context-dependent, as ants doing midden work tended to remove oleic acid-treated papers to the midden, while ants that were foraging tended to carry the papers to the nest, treating them as food items (Gordon, 1983).

Interestingly, oleic acid has also been identified as a major elaiosome component (Brew *et al.*, 1989; Kusmenoglu *et al.*, 1989; Lanza *et al.*, 1992). Thus, speculation ensued about its potential role in seed-dispersal induction. In four known myrmecochores, *Jeffersonia diphylla*, *Sanguinaria canadensis*, *Trillium sessile*, and *Dicentra cucullaria*, high levels of oleic acid were found, along with high levels of palmitic acid and triacylglycerols and slightly lower levels of linoleic acid (Kusmenoglu *et al.*, 1989). Similarly, in *Trillium erectum*, *T. grandiflorum*, and *T. undulatum*, oleic acid was followed in abundance by linoleic and palmitic acids (Lanza *et al.*, 1992).

Other workers have accumulated evidence that the diglyceride diolein is involved in inducing the seed-carrying behavior. Marshall *et al.* (1979) found that the primary active portion of the elaiosomes of *Viola odorata* was 1,2 diolein while the 1,3 isomer elicited little seed-carrying by *A. rudis*. Although free fatty acids elicited seed-carrying, it was found to be only 60% as effective in eliciting seed-carrying behavior as the diglyceride. In similar bioassays, Marshall *et al.* also found that intact seeds, isolated elaiosomes, and the diglyceride fraction showed the same level of activity, indicating that the free fatty acids do not add significantly to the ants' responses. Using bioassays, Skidmore and Heithaus (1988) found that *Pogonomyrmex rugosus* did not differentiate between isolated elaiosomes of *Hepatica americana* and inert cubes coated with the diglyceride fraction of the elaiosome. They also found that the ants responded less intensely to cubes coated with the free fatty acid fraction, and that diolein was a major component of the diglyceride fraction, supporting the work of Marshall *et al.* (1979).

Further behavioral experiments by Brew *et al.* (1989) showed that inert pieces of pith treated only with the unsaturated oleyl-based lipids of the elaiosome (oleic acid, 1,2-diolein, and triolein) were removed at rates comparable to those of the elaiosomes of *Acacia myrtifolia* and *Tetralthea stenocarpa*. Although untested, linoleic acid was obviously unnecessary for removal. Brew *et al.* also showed that the chemical configuration of the oleyl-groups was important, as piths treated with 1,3-diolein were removed at significantly lower rates than pith treated with 1,2-diolein, giving further support to the conclusions of Marshall *et al.* (1979).

With this background, we investigated the sensory bases of seed carrying by ants using behavioral, physiological, and morphological approaches. We initially investigated the roles of olfaction and gustation in seed-carrying behavior using a behavioral assay as some studies have described the ants as being "attracted" to either the seeds with elaiosomes or to the chemical components

in the elaiosomes (Marshall *et al.*, 1979; Hanzawa *et al.*, 1985). In contrast, Gunther and Lanza (1989) have described the ants' behavioral sequence as consisting of antennation of the seed, followed by chewing, picking up, and the removal of the seed, while others have also noted that contact with the seed was necessary for seed-carrying (O'Dowd and Hay, 1980; Kjellsson, 1985). It is conceivable that both olfaction and gustation could be happening in concert, with the behavioral sequence following the initial "attraction". Second, we used electroantennograms (EAGs) to investigate further the sensory bases for the behavioral data and to provide information about physiological thresholds for chemical perception: EAGs have already been successfully employed to show the differing antennal responsiveness of ants to different pheromones and analogues (Payne *et al.* 1975; Glancey and Dickens, 1988; Andryszak *et al.*, 1990; Kern and Bestmann, 1993). Third, we studied the ant antennae using the scanning electron microscope (SEM) to investigate the receptor(s) involved in seed dispersal induction. Ant antennae possess 5–8 general types of sensilla (Hashimoto, 1990) including sensilla basiconica (implicated in olfactory reception); sensilla chaetica (implicated in contact chemosensory or mechanosensory reception); sensilla placodea/sensilla trichodea curvata (implicated in olfactory reception); sensilla coeloconica (implicated in olfactory reception) and sensilla ampullacea (implicated in temperature, humidity or CO<sub>2</sub> reception). The types of sensilla in a given ant, then, can provide a clue to the behavioral and physiological capabilities of the ant.

Our behavioral results indicate that the ants used in our study disperse *A. canadense* seeds only after chancing upon them and interacting with them according to Gunther and Lanza's (1989) "antennate, chew, pick-up, remove" model. As in Kjellsson's (1985) study, sight and smell were not sufficient for the localization of seeds by our ants. EAG recordings further support a gustatory discrimination mechanism: olfactory delivery of increasing dosages of mixed isomer diolein produced antennal responses no different from controls while contact delivery produced dose-dependent responses. The SEM revealed the presence of both olfactory and contact chemoreceptors, the latter of which appear to be involved in mediating seed-carrying.

## MATERIALS AND METHODS

### *Behavioral assays*

*Animals.* Fourteen ant colonies from 4 different species were located using small chunks of oil-packed canned tuna as bait (Beattie and Culver, 1981) in a second-growth mixed hardwood forest north of Gambier in Knox County, Ohio, during July 1991. We studied the behavior of individuals from: *A. rudis* Emery, 8 colonies; *Lasius alienus* (Foerster), 2 colonies; *Formica subsericea* Say, 2 colonies; *Camponotus ferrugineus* (F.), 2 colonies. The ants were identified by D. R. Smith, Systematic

Entomology Laboratory, USDA. At our study site, the species studied are generalists, feeding on insects, honeydew, and elaiosomes (E. R. Heithaus, pers. comm.).

*Seeds with elaiosomes.* The seeds of *Asarum canadense* used in the behavioral experiments were harvested in mid-June, 1991, several weeks before our assays. The seeds were kept frozen (–20°C) until use. When frozen, *A. canadense* seeds maintain their ability to elicit carrying by ants for at least one year (E. R. Heithaus, pers. comm.).

*The olfactory assay.* Ants were tested for olfactory response by measuring the time spent by individual ants on each of two 5.5 cm Whatman #1 filter paper circles placed within 10–15 cm of the nest opening. The filter papers were equidistant from the nest opening, and had a small hole punched in the center using a hole punch. A small white opaque plastic capsule (BEEM-type, Ted Pella Co.), in which eight 1 mm dia. holes were drilled, was inserted into the hole of each filter paper so that the perforated portion of the capsule protruded from the plane of the paper. In the experimental treatment, the capsule contained three wild ginger (*A. canadense*) seeds with elaiosomes, while in the control treatment the capsule was empty. We then watched the two filter paper circles over 30 min observation periods during the late morning to the mid-afternoon, timing the duration that individual ants spent on each filter paper using a stopwatch. If ants are capable of detecting the seeds by olfaction, and are attracted to them, we would expect ants to spend more time in the test circle containing the capsule with the seeds than the circle with the empty capsule. For the both treatments, we also noted the ant's behavior, especially if the ant managed to antennate the inside of the capsule by inserting its antennae into the holes. Data were statistically analyzed by species using ANOVA and Student's *t*-test on the Minitab statistical package.

*The gustatory assay.* Gustatory response was tested in a manner similar to that of the olfactory response. Again, we measured the time spent by individual ants on each of two 5.5 cm Whatman #1 filter paper circles placed within 10–15 cm of the nest. In this series of tests, however, a single wild ginger (*A. canadense*) seed was affixed with cyanoacrylate glue (Elmer's Wonderbond, Borden) to the center of one circle, while the other circle remained empty. We again observed the ants' behavior, classifying it according to whether the ants antennated or touched the seed at the center of the filter paper disk. Data were analyzed by species using ANOVA (Minitab).

### *Physiological assays*

*Animals.* Three colonies of *F. subsericea* Say initially used for the behavioral assays were collected and brought into the laboratory for the physiological investigations. The colonies were maintained in the lab on an artificial diet (Hölldobler and Wilson, 1990) in plastic boxes (32 × 24 × 10 cm, Tri-State Plastics) with plaster bottoms. The sides of these boxes were coated with Fluon (Northern Products Inc.) to prevent escape, while a foil-

covered test tube stuffed with moist cotton served as an artificial nest. We also brought colonies of *A. rudis* Emery and *L. alienus* (Foerster) into the laboratory for the EAGs, but we found that their small size made consistent recordings from their antennae in the contact chemosensory assays very difficult.

**Olfactory EAG recordings.** We recorded olfactory responses to the diolein stimulus using standard protocol (Roelofs, 1984). Ants observed to exhibit foraging behavior were cold-anesthetized in a freezer for 10 min before their heads were isolated and mounted on a Sylgard (Dow-Corning) block using minuten pins (Carolina Biological). The most distal antennal segment of one antenna was removed with scissors, and a glass electrode pulled on a horizontal puller (Sutter Instruments model P-87) was manipulated over the cut antennal tip using a micromanipulator. The electrode was filled with receptor lymph saline (Kaissling and Thorson, 1980) to complete the electrical circuit. An AgCl wire was inserted into the ant head to serve as the indifferent electrode. The massed receptor potential was amplified using a DC electrometer (Gettling model 5A); viewed on a storage oscilloscope (Tektronix model 5111 or 2201); and recorded on magnetic tape on an instrumentation recorder (Vetter model B). Hardcopy of the responses were made using a chart recorder (Gould model 3200).

**Olfactory stimulation.** For olfactory stimulation, the antenna was continuously ventilated with charcoal-filtered humidified air (0.7 l/min) through a glass cartridge (1/2 cm<sup>3</sup> syringe, Becton-Dickinson Glaspak) containing a 5 × 5 mm piece of Whatman #1 filter paper rolled into a tube. A second glass cartridge containing filter paper of the same size soaked with the stimulant was also directed at the antenna at an angle of about 15° with respect to the first cartridge. Both cartridges were about 5 cm away from the preparation. During olfactory stimulation, the antenna was stimulated for 1 s by switching the airflow from the blank cartridge to the odorant cartridge using a solenoid (General Valve) controlled by a physiological stimulator (Gettling model DS1). Odorant cartridges were prepared daily from serial dilutions (10<sup>-1</sup>–10<sup>-7</sup>) of a stock solution (25% v/v) of mixed-isomer (1,2 and 1,3) diolein (Sigma Chemical #D8894) diluted in *n*-hexane (J.T. Baker, GC-grade). 10 μl aliquots were applied to each filter paper square and inserted into the odorant cartridges after drying. The antenna was stimulated with odorants at 1 min intervals, with the odorants being applied in order of increasing concentration. The responses to the odorant were compared with the response to 10 μl of *n*-hexane carrier, which served as the control.

**Contact EAG recordings.** To record the EAGs in response to contact application of diolein, the ant was anesthetized and the head was isolated according to the protocol described earlier. The head was affixed to the inside of an "L"-shaped block of Sylgard using minuten pins, and one antenna was elevated alongside the vertical face of the block (Fig. 1). The raised antenna was kept

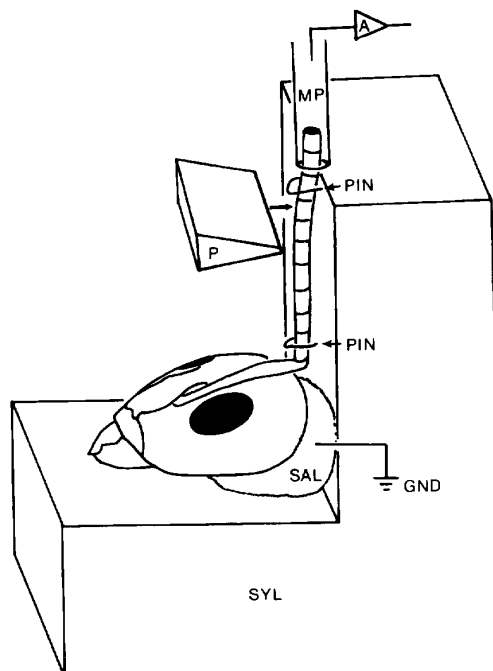


FIGURE 1. A schematic diagram of the preparation used for recording EAG responses to contact application of diolein. SYL, Sylgard block; SAL, saline droplet; GND, preparation ground; PIN, minuten pin loops for affixing antenna; P, polyphorous strip containing the stimulus; MP, recording micropipette; A, lead to the amplifier. The ant head was pinned to the Sylgard using minuten pins. These pins, the other antenna, and the micromanipulator used to maneuver the pith are not shown.

in place using 2 small, "U"-shaped minuten pins, and the most distal segment was removed using scissors. A glass electrode filled with receptor lymph saline was maneuvered over the cut end of the antenna, and the ground AgCl wire was inserted into the saline at the base of the head. The EAGs were amplified and recorded according to the protocol described earlier.

**Contact stimulation.** For contact stimulation, the diolein was applied to an inert polyphorous strip (BioQuip Co.), whose edge was roughly the size of an elaiosome. The stock 10 cm long polyphorous strips were shaped using a razor blade to form a wedge about 1 mm thick at the back, tapering to a thinner edge along the entire length of the strip (Fig. 1). The width of the wedge was about 3 mm. Using razors, triangular notches were cut from the tapered edge of the wedge at regular intervals, so as to produce 3 mm long "teeth" along the entire edge of the wedge. The back of the wedge was then glued (Elmer's Wonderbond, Borden) to a thin piece of balsa wood that could be manipulated by a micromanipulator (Leitz). A 5 μl aliquot of a serial dilution (10<sup>-1</sup>–10<sup>-12</sup>) of the mixed diolein stock solution (see earlier protocol) was then applied to each 3 mm wide "tooth" along the polyphorous strips so that the stimulant concentration increased with each "tooth" along the strip. The "teeth" limited the diffusion of each aliquot so that each "tooth"

contained diolein of only one concentration. The control treatment was 5  $\mu$ l of the *n*-hexane.

To stimulate, the strip of "teeth" with the balsawood backbone was maneuvered using the micromanipulator so that a "tooth" could be brought into gentle contact with the antenna for 1–1.5 s (see Fig. 1). Typically, we stimulated the antenna in the region of segments 5–9 at 1 min intervals, moving the strip so that the diolein was applied to the antenna in increasing concentration from the control stimulus.

Our EAG recordings are complicated somewhat by the fact that the last antennal segment, which is well-endowed with sensory receptors, is removed in the course of the recording. Hence, neither our olfactory nor contact application of diolein stimulated the entire sensory complement of the antenna. In observing the antennation of elaiosomes by freely behaving ants, it appeared that the last few segments of the antenna were preferentially used to assess the elaiosome. We therefore applied the diolein as near the tip as possible to mimic the actual behavior. However, due to the difficulty of moving the stimulating wedge onto the antenna, then off it, we could not imitate the quick palpations that were observed in antennating ants.

#### Morphological studies

Antennae from two individuals each of *A. rudis* Emery, *F. subsericea* Say, and *C. ferrigineus* (F.) were examined by SEM. Isolated heads of these ants were air-dried, and then sputter-coated (Polaron Equipment model E5100) with platinum 100–125 Å thick. The antennae were then viewed using a SEM (ISI model 40) at the Ohio Agricultural Research and Development Center, Wooster, Ohio.

## RESULTS

#### Behavioral assays

Ants showed no indication that they were able to perceive the diaspores based solely on air-borne cues. In all 4 species, (*A. rudis* Emery; *F. subsericea* Say; *C. ferrigineus* (F.); *L. alienus* Foerster) we did not find significant differences in the times spent within a test circle as long as the ants did not antennate the inside of the plastic capsule through the small holes (Table 1). If antennation occurred, then the time spent in the test circle rose dramatically. This was most evident for *A. rudis*, from which the sample size was the largest. The results of the gustatory assay supported the findings from the olfactory assay. Ants that antennated the diaspores spent significantly more time in the test circle than those that entered the blank test circles or those that entered the experimental circles without antennating the diaspore (Table 2). We did not see evidence that the ants were visually guided to the diaspores as they routinely walked within several centimeters of the dark diaspore on the white test circle without investigating the diaspore. In contrast, if the dia-

spore was antennated, the ants would often spend a great deal of time either trying to pull the diaspore out of the holes in the capsule, or trying to pull the Super-glued diaspore from the test circle.

The mean time spent in the test circle by ants not antennating the diaspores varied somewhat between ant species, depending on the typical walking speed of each species. Larger ants, such as *C. ferrigineus*, walked more quickly than the smaller species, consequently spending less time in passage across the test circle. Across both assays and all ant species, the mean time ( $\pm$ SEM) spent in circles where no seeds had been placed was  $6.4 \pm 0.7$  s. For circles where the seeds had been placed, but not antennated, the time was  $6.1 \pm 1.0$  s. In contrast, if the seed was antennated, ants spent  $129.1 \pm 23.4$  s.

In *A. rudis*, we were able to calculate the contribution of both treatment and colony effects on the total variance of times spent in the test circles. In the "olfactory" assay, colony effects accounted for 3% of the total variance, and the treatment for 58%. In the "gustatory" assay, colony effects accounted for 2% and the treatment for 26% of the total variance.

#### Electroantennogram assays

The EAG assay produced data supporting the behavioral data. Olfactory applications of dilutions of mixed isomer diolein to the antennae of *F. subsericea* Say produced responses no different from the mechano-sensory response to the hexane blank cartridge (Figs 2, 3). Contact application of mixed diolein dilutions, however, produced dose-dependent responses that were consistently larger than that to the hexane blank (Figs 4, 5). The response appeared to peak at about  $-5$  log dilution of the diolein, with the variance of the response increasing with the application of higher diolein concentrations.

#### Scanning electron microscopy

Morphological analysis of the antennae of *F. subsericea* Say, *C. ferrigineus* (F.), and *A. rudis* Emery revealed very different distributions and types of sensilla. While the antennae of *F. subsericea* and *C. ferrigineus* are quite densely covered with sensilla (Figs 6–10), the antennae of *A. rudis* have far fewer sensilla (Fig. 11). The common denominator in all three species was the dominant presence of thin, curved bristles, or sensilla chaetica, which are believed to have a mechanosensory and/or contact chemosensory function (Hashimoto, 1990). In *A. rudis*, the bristles are supplemented by longer hairs emanating from sockets on the antennal surface (sensilla trichodea; Fig. 11) that have been identified as being olfactory receptors (Hashimoto, 1990). In *F. subsericea* and *C. ferrigineus*, however, we saw two additional types of sensilla: a thick sensillum that originates from a socket and lies flat in a groove against the antennae (sensilla placodea; Fig. 7); and a short, thick curved sensillum whose base rests within a groove (sensilla trichodea curvata; see Figs 6, 10). Both are believed to have an olfactory function (Hashimoto, 1990). Sensilla placodea were

TABLE 1. Time spent by ants in test circles with perforated capsules with or without diaspores. [Mean in s  $\pm$  SEM (sample size)]

Ant species	Capsules with diaspores			P value
	Capsules without diaspores	Ant did not touch diaspore	Ant touched diaspore	
<i>A. rudis</i> Emery	8.1 $\pm$ 1.5 (37)	9.6 $\pm$ 2.4 (31)	136.7 $\pm$ 20.8 (10)	0.000*
<i>F. subsericea</i> Say	6.3 $\pm$ 1.2 (28)	5.4 $\pm$ 1.1 (20)	—	0.58 †
<i>C. ferrigineus</i> F.	1.5 $\pm$ 0.3 (14)	2.9 $\pm$ 0.8 (19)	37.3 (1)	0.14 †
<i>L. alienus</i> Foerster	4.0 $\pm$ 1.0 (7)	4.2 $\pm$ 1.4 (8)	15.9 (1)	0.91 †

\*One-way ANOVA

†Two-way *t*-test: Due to the small or non-existent number of ants that touched diaspores in the capsules, a *t*-test was performed on the data from the first two data columns.

TABLE 2. Time spent by ants in test circles with or without diaspores at the center. [Mean in s  $\pm$  SEM (sample size)]

Ant species	Diaspore at the center			P value
	No diaspore	Ant did not touch diaspore	Ant touched diaspore	
<i>A. rudis</i> Emery	6.6 $\pm$ 1.3 (16)	4.0 $\pm$ 1.3 (6)	97.7 $\pm$ 36.6 (8)	0.001*
<i>F. subsericea</i> Say	26.0 $\pm$ 6.1 (2)	5.0 $\pm$ 3.9 (2)	296 $\pm$ 101 (3)	†
<i>C. ferrigineus</i> F.	3.3 $\pm$ 0.8 (2)	1.5 (1)	8.5 (1)	†

\*One-way ANOVA

†Insufficient sample size for statistical analysis.

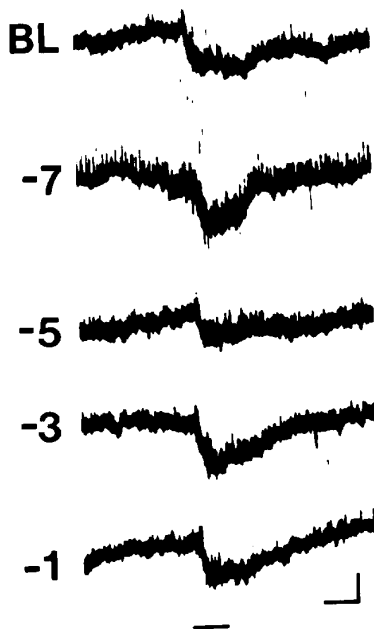


FIGURE 2. The EAG responses from a single individual of *Formica subsericea* Say to olfactory stimulation using varying doses of mixed isomer diolein. BL, response to a hexane blank cartridge; -7, -5, -3, -1, responses to decreasing log dilutions of mixed diolein. Stimulus bar = 1 s. Scale bars = 1 mV (vertical) and 1 s (horizontal).

confined to the middle 5 or 6 antennal segments: segments 3–8 in *F. subsericea* and segments 3–7 in *C. ferrigineus*. Sensilla trichodea curvata, on the other hand, were not confined to specific segments.

## DISCUSSION

Our behavioral experiments show that the ants used in our study perceive *A. canadense* seeds by contact or very close-range chemoreception, rather than by long-range olfaction. We saw little evidence that the ants of 4 species are attracted to the *A. canadense* diaspores, either by olfactory or visual cues, but it appears that they chance upon them. We found no difference in the time spent in the test circles by ants in different treatments as long as the diaspores were not antennated. Once the ants appeared to antennate the diaspores, we found that their behavior took a dramatic turn as they tried to remove the diaspores, spending 12–15 times more time in the test circle as compared to the ants that did not antennate.

The small number of ants responding to the diaspores may also be explained by polyethism among the workers. Hence, our results could also be due to the fact that few foraging workers encountered the seeds, while other ants with different behavioral roles did not respond to the seeds after encountering them. The differences in response among workers with different behavioral roles could be due to both central mechanisms, as well as dif-

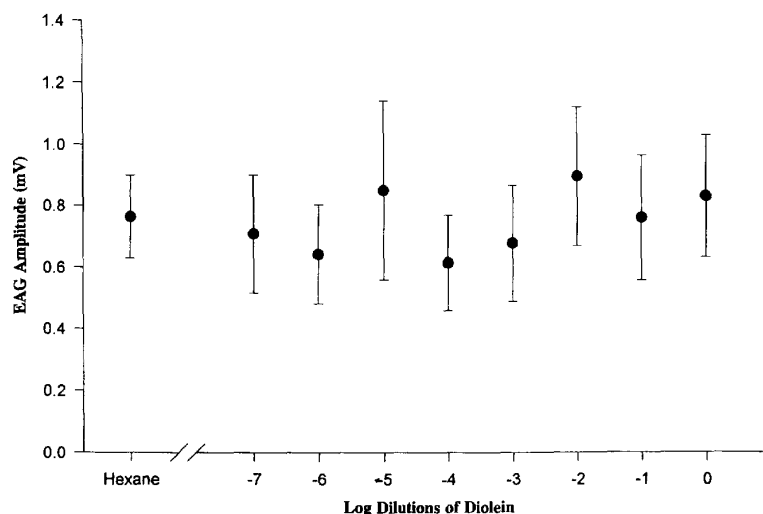


FIGURE 3. The mean EAG responses ( $mV \pm SEM$ ) from *Formica subsericea* Say in response to olfactory application of log dilutions of mixed diolein and to a hexane blank. Each data point is the mean of 4 individuals.

ferences in sensory complements among ants with different behavioral roles.

Our physiological data support our behavioral findings: olfactory delivery of increasing dosages of mixed isomer diolein produced results no different from the mechanosensory response to a blank cartridge. However, contact delivery produced responses that increased with dosage. The variability of the response increased with the higher concentrations, perhaps indicating that some of the receptors were habituating, and require longer inter-stimulus intervals than we used in order to recover fully. The variability could also be exacerbated by differences in the exact shape of the polyporous teeth that were used to apply the diolein to the antenna. However, if this were the case, we would expect the variability to be dispersed randomly across all the different dilutions: something that we did not see. Another possible source of variation

is due to polyethism. For our physiological studies, we attempted to select foraging ants from our small laboratory containers. However, ants with different behavioral roles might have been outside the nest, and the different antennal sensitivities could be associated with these different behavioral roles. Gordon (1983) has demonstrated that the behavioral responses of an individual *Pogonomyrmex badius* to oleic acid is dependent on the social role (forager, midden worker, etc.) of that individual ant. It is possible that some of the differences in behavior could be due to differences in antennal sensitivity to behaviorally relevant chemicals.

The behavioral and EAG data indicate that the ants studied need to be extremely close, if not touching the *A. canadense* diaspore, to be able to recognize it. We do not yet know if contact between the diaspore and the antenna is actually necessary for an antennal response. Very close range olfactory perception could explain why some ants stuck their antennae through the perforations in the seed-containing capsule during the olfactory behavioral studies. In support, Gunther and Lanza (1989) note that no *Myrmica punctiventris* in their study ignored *Trillium* seeds if they passed within 1cm of the seeds. In contrast, Kjellsson (1985) found that direct contact of *Carex pilulifera* seeds was necessary before individual *Myrmica ruginodis* would carry the seed. As well, Städler and Hanson (1975) report that contact chemoreceptors in the larvae of the sphinx moth *Manduca sexta* have olfactory capabilities at extremely close ranges, as long as the stimulus is strong enough. In the ants studied here, it may be that very close range olfaction on the order of 1mm exists (via olfactory or gustatory receptors), but longer-range olfaction was not supported by our observations.

The lack of a long-range olfactory mechanism may be explained by the fact that the chemicals implicated in

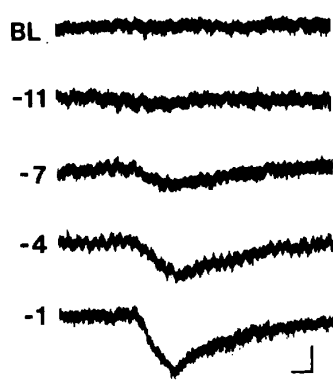


FIGURE 4. The EAG responses from a single individual of *Formica subsericea* Say to contact stimulation using varying doses of mixed isomer diolein. BL, response to a hexane blank cartridge; -11, -7, -4, -1, responses to decreasing log dilutions of mixed diolein. Stimulus bar = 1.5 s. Scale bars = 1 mV (vertical and 1 s (horizontal)).

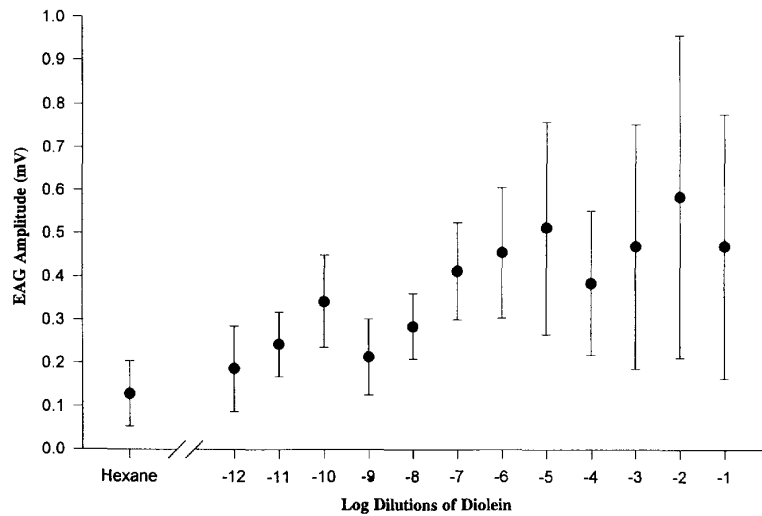


FIGURE 5. The mean EAG responses ( $mV \pm SEM$ ) from *Formica subsericea* Say in response to the contact application of log dilutions of mixed diolein and to a hexane blank. Each data point is the mean of 7 individuals.

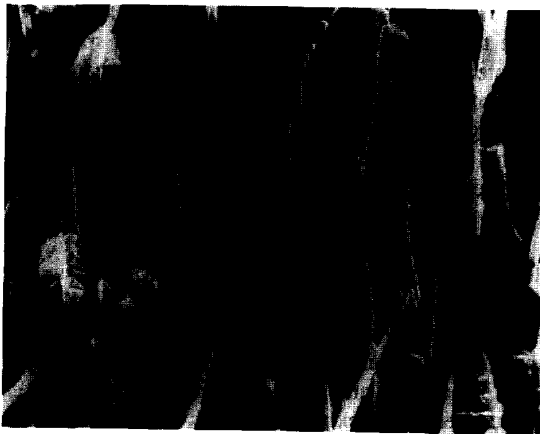


FIGURE 6. Scanning electron micrograph of the terminal (12th) antennal segment of a *Formica subsericea* Say. Note the *sensilla trichodea curvata* (arrows) and the bristles, or *sensilla chaetica* (nomenclature according to Hashimoto, 1990). Scale bar = 5  $\mu m$ .



FIGURE 7. Scanning electron micrograph of the 8th antennal segment of a *Formica subsericea* Say. Note the *sensilla placodea* (arrows; Hashimoto, 1990). Scale bar = 25  $\mu m$ .

seed-carrying, namely oleic acid ( $C_{18}H_{34}O_2$ ), 1,2-diolein ( $C_{39}H_{73}O_5$ ), and triolein ( $C_{57}H_{104}O_6$ ) (Skidmore and Heithaus, 1988; Brew *et al.*, 1989), are all long-chain hydrocarbons with low volatilities. Among the Lepidoptera, whose aliphatic female sex pheromones have been well-characterized, investigators have found that carbon chain lengths of 14, 16, and 18 are the most common within the observed range of 12–21 carbons (Chapman, 1982). For diolein and triolein, whose chain lengths are substantially longer, we would expect their volatilities to be substantially less than that of oleic acid due to the increased sizes of the molecules (Agosta, 1992). However, it should also be noted that many insect pheromones with relatively low volatilities are still perceived by olfactory receptors in minute quantities. Investigators performing olfactory EAGs on male moths, for example, are typically able to detect nanogram quantities of a female sex



FIGURE 8. Scanning electron micrograph of the 6th antennal segment of a *Camponotus ferrugineus* (F.). Scale bar = 25  $\mu m$ .





FIGURE 9. Close-up scanning electron micrograph of the 6th antennal segment of a *Camponotus ferrigineus* (F.). Note the *sensillum placodea* as well as bristles. Scale bar = 5  $\mu$ m.

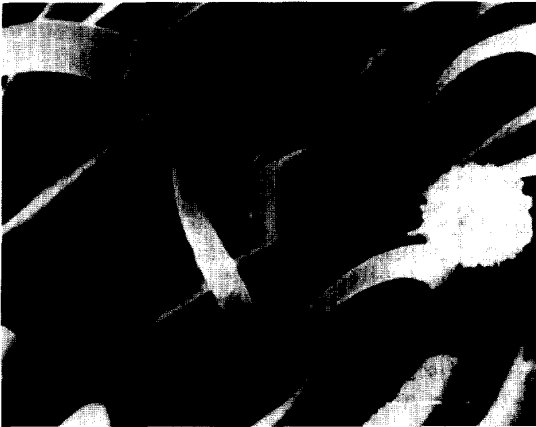


FIGURE 10. Close-up scanning electron micrograph of the 6th antennal segment of a *Camponotus ferrigineus* (F.). Note the *sensillum trichodea curvata* as well as bristles. Scale bar = 5  $\mu$ m.



FIGURE 11. Scanning electron micrograph of the 4th antennal segment of a *Aphaenogaster rudis* Emery. Scale bar = 10  $\mu$ m.

pheromone (e.g. Christensen and Hildebrand, 1987), while recent work on the olfactory EAG responses to trail pheromones from the ants *Atta texana* and *Leptogenys diminuta* show threshold responses in the low  $\mu$ g range (Andryszak *et al.*, 1990; Kern and Bestmann, 1993). It is very possible that we did not use a large enough dose of the diolein in our olfactory EAG assays to be able to record a response. However, based on the behavioral evidence, it appears that seeds of *A. canadense* are not perceived via long-range olfaction. Thus, to use a high enough dose of diolein to get measurable olfactory EAG response may be to use a dose that is far beyond that actually seen in nature. There may also be a parallel situation in the trail pheromone of *A. texana* where the behavioral threshold for trail following is 4.0 pg, with suppression of the trail-following behavior above 0.4 ng (Robinson *et al.*, 1974) while the EAG threshold for olfactory detection was in the range of 10  $\mu$ g (Andryszak *et al.*, 1990).

In our case, the situation is complicated by the fact that no study has directly measured the actual diolein content in different elaiosomes, although the ratios of different fatty acids have been determined for 3 species of *Trillium* (Lanza *et al.*, 1992). Our work clearly indicates, however, that the contact application of diolein results in an EAG response at diolein concentrations that are insufficient to produce an olfactory response. Based on our data, we cannot distinguish between very close-range olfactory and/or contact chemoreception as the mechanism by which ants perceive the elaiosomes. To differentiate between close-range olfaction and contact perception, it is necessary to use close-range video analysis of the ants' behavior close to the seed in conjunction with careful physiological studies, looking at whether diolein must be in contact with the antenna in order to generate EAG responses. Better yet, recording from individual olfactory and contact chemoreceptors would clarify which modality is actually mediating the response. Single unit recordings of olfactory receptors from the antenna of *Lasius fuliginosus* (Latr.) have been done by Dumpert (1972), but recording from individual gustatory receptors while stimulating it will be much more difficult.

Morphological analyses of *F. subsericea*, *C. ferrigineus* and *A. rudis* antennae revealed very different distributions and types of sensilla. Sensilla trichodea curvata and/or sensilla placodea, which have olfactory function (Dumpert, 1972; Hashimoto, 1990) were found in all 3 species. The sensilla chaetica (or bristles) that have been implicated in contact chemoreception were also found in large numbers in all species (Hashimoto, 1990). Thus, the SEM has shown us that each species appears to possess both the contact and the olfactory chemoreceptors on their antenna necessary to explain the behaviors and the physiological results. One explanation for the lack of an EAG response to the olfactory delivery of diolein is that the last segment, which was removed for the EAG recording, contained all or most of the olfactory receptors. The presence of olfactory sensilla on antennal seg-

ments other than the last indicates not all olfactory receptors were removed to make the EAG recordings. However, we do not know the selectivity of the olfactory receptors along the antenna, so it is possible that diolein-responsive cells are preferentially located at the tip of the antenna.

A confounding factor in this study is the context in which the ants studied encountered the elaiosomes. The *A. canadense* seeds used in the behavioral assays, typically, are found several weeks before the dates of our behavioral assays, so that ants walking by a seed in our assay may not be expecting to encounter it. However, during the period in which our behavioral assays were performed, other myrmecochores, such as *Trillium* spp., are dropping their seeds. Other behavioral assays have shown that ants found at our study site are willing to carry *A. canadense* seeds weeks after they are normally found in the field (E. R. Heithaus, pers. comm.).

Another factor that may play a role in our study are the seed preferences of the ant species studied. There is little doubt that some ant species are more likely than others to move seeds (Bossard, 1981; Davidson and Morton, 1981; Kjellsson, 1985; Horvitz and Schemske, 1986b; Mossop, 1989; Oostermeijer, 1989). It is also possible that ant species studied prefer seeds other than *A. canadense* that were present during the study (or may not carry seeds at all), thereby affecting the results of our behavioral assay. For example, Oostermeijer (1989) showed that ants of 3 species showed preferences among seeds of 3 myrmecochores. However, all the ant species studied here are known to carry *A. canadense* seeds at the study site (E.R. Heithaus, pers. comm.), and *Aphaenogaster* spp., *F. subsericea*, and *L. alienus* have been shown to disperse *Viola* seeds in West Virginia in a similar habitat (Culver and Beattie, 1978). As well, those ants that antennated the seeds in our behavioral assays typically showed great interest in them, often spending a good bit of time trying to remove the seeds.

Our results indicate that contact chemoreception appears to be the most important factor in mediating seed-carrying of *A. canadense* seeds in the ant species studied. Our results support Gunther and Lanza's (1989) view of seed choice by ants as a four-step process consisting of antennation of the seed, followed by chewing, picking up, and the removal of the seed. Furthermore, we have found that diolein is perceived by contact or very close-range chemoreception, supporting the conclusions of other investigators (Marshall *et al.*, 1979; Skidmore and Heithaus, 1988; Brew *et al.*, 1989) that it plays a role in seed-carrying behavior. While we can say little about the sensory bases of seed removal by other ants such as harvester ants, for those seeds in which diolein plays a role in dispersal, we believe that the ants are using gustation rather than olfaction to detect the diolein. We are now progressing to studies that address some of the questions that have been raised by this paper.

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