examination of fluids. My experiments with capillaries showed optical aberrations too great for clear resolution of bacteria, but a wet mount with paired cover slips or a cover slip and slide was acceptable. I used a simple Bausch and Lomb Nicholas spotlight illuminator as the light source; Leeuwenhoek used sunlight or a candle, sometimes in conjunction with magnifying mirrors to concentrate and direct the light. For the Leitz Orthoplan, I placed the light source beneath the front of the microscope stage with the light aimed at the sample. The light beam was at an angle of 45° with the plane of the sample (135° with the optical axis of the microscope). Suspensions of *Escherichia coli*, *Arthrobacter globiformis*, and nonsporulated *Bacillus megaterium* were examined in this manner. At 100- and 250-fold magnifications, the cells were easily seen (Fig. 1) because they were brightly illuminated against a black background as in dark-field illumination. This was true for cells in suspensions as well as those attached to the glass. Similar lighting was used for the Leeuwenhoek microscope and the other magnifiers, and it was found that dark-field illumination occurred at lighting angles of 45° or less, but that illumination of the cells decreased correspondingly as the angle was decreased from this value. Fungal mycelium (*Mucor* species) was clearly seen as dark-field with all magnifiers, including the Leeuwenhoek scope. A bacterium, *Bacillus mycoides*, that had not sporulated was observed as dark-field with the Leeuwenhoek microscope; the cells were seen coming into and out of the focal plane as they flowed past the field being viewed. This observation, including the appearance of the cells, was checked by observing the preparation with a phase microscope.

For Leeuwenhoek to have used this procedure would merely have required a beam of sunlight or candlelight striking his sample from behind at an angle of 45°. He may, however, have concentrated and aimed the beam with his magnifying mirrors. In addition to this, I suggest that he may have used his microscopes of lower power—that is, those magnifying in the range of 100 to 150 diameters—to observe suspended bacteria. These would have provided him with a greater depth of field for obtaining focus as compared to his instruments of greater magnifying power.

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## Ant-Aphid Association: Role of Aphid Alarm Pheromone

Abstract. *When attacked by predators, aphids secrete alarm pheromones that cause nearby aphids to disperse. Ant-associated (myrmecophilous) aphid species disperse less readily than nonmyrmecophilous species. Ant exposure further depresses the dispersive alarm behavior of myrmecophilous species. The ant* Formica subsericea *responds to aphid alarm pheromone in a way that is beneficial to the aphid. These findings support our hypothesis that myrmecophilous aphids depend more on ants for protection from predators than on their own dispersive powers.*

Aphids are small, soft-bodied insects that feed on plants. Their sedentary, gregarious habits make them particularly vulnerable to predator attack. Aphids have countered this threat by evolving a dual, self-serving and altruistic defensive system involving the aphid's cornicle secretion. When attacked by predators, aphids secrete cornicle droplets (Fig. 1). These "sticky" droplets, composed largely of triglycerides, can impede an attacking predator and result in the release of aphid prey (*1*). The droplets also contain hydrocarbons, notably *trans*-β-farnesene, which serve as alarm pheromones that inform nearby aphids of impending danger. Aphids then fall, jump, or walk away to escape (*2*).

Certain aphid species are also protected from predators by the ant species that tend them (*3*). In field studies, we found that ant-associated (myrmecophilous) aphids disperse less readily to alarm pheromones than do nonmyrmecophilous species. We suspected that myrmecophilous aphids may depend more on ants for protection than on their own dispersive powers. We tested this hypothesis under controlled conditions in the laboratory and found a marked dif-

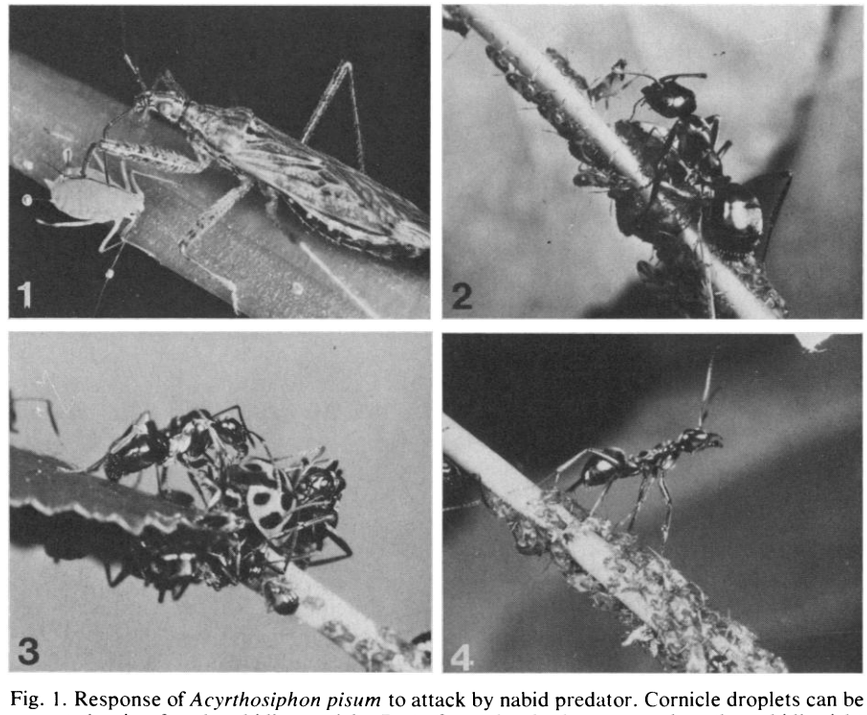


Fig. 1. Response of *Acyrthosiphon pisum* to attack by nabid predator. Cornicle droplets can be seen at the tip of each aphid's cornicle. Part of one droplet has smeared on the aphid's right antenna. Fig. 2. *Chaitophorus populicola* standing in response to antennation by *Formica subsericea*. Continued antennation results in excretion of a honeydew droplet that is collected by the ant. Fig. 3. Ants are shown attacking a coccinellid aphid predator by biting with mandibles and smearing contents of Dufour's gland from abdominal tip. Fig. 4. An ant responds to *trans*-β-farnesene by raising and extending antennae, opening mandibles, and orienting toward pheromone source.

Table 1. Response of myrmecophilous and nonmyrmecophilous aphids to alarm pheromone. S, strongly myrmecophilous; W, weakly myrmecophilous; N, nonmyrmecophilous.

| Aphid: tribe and species | Falling (%) in response to alarm pheromone |
|---|---|
| *Subfamily Aphidinae* | |
| Aphidini | |
| *Aphis fabae* (S) | 1.3▲ |
| *Rhopalosiphum padi* (W) | 61.6■● |
| *Schizaphis graminum* (W) | 98.5● |
| *Hyadaphis erysimi* (N) | 51.1■ |
| Macrosiphini | |
| *Macrosiphum euphorbiae* (N) | 51.1■ |
| *Acyrthosiphon pisum* (N) | 93.5● |
| *A. solani* (N) | 39.4■ |
| *Myzus persicae* (N) | 43.2■ |
| *Subfamily Chaitophorinae* | |
| Chaitophorini | |
| *Chaitophorus viminalis* (S) | 0 ▲ |
| *C. populicola* (S) | 0 ▲ |
| Siphini | |
| *Sipha kurdjumovi* (N) | 80.5● |

*No significant difference ($P < .05$, Student-Newman-Keuls test; multivariate analysis) between percentages labeled with the same symbol (▲, ■, or ●).

ference in alarm behavior between myrmecophilous and nonmyrmecophilous aphids. Furthermore, we found that ants alter the alarm behavior of myrmecophilous aphids and that ants respond to aphid alarm pheromone.

Although it is generally accepted that there is an association between certain aphid taxa and ants that attend them (*4*), lists of aphid species attended by ants are lacking. From two aphid subfamilies we selected 11 species (*5*) for study to determine which were myrmecophiles. After exposing colonies of these aphids to the aphidicolus (aphid-tending) ant, *Formica subsericea*, the aphid-ant interaction was observed (*6*). Ants antennated (stroked aphid's bodies with their antennae) or attempted to antennate all aphid species (Fig. 2). Three species responded by readily excreting honeydew droplets, two species responded only infrequently, and six species did not respond at all (Table 1). The Macrosiphini species were disturbed by the ants and dispersed. This triggered a predatory response by the ant and these aphids were attacked, carried to the ant nest, and presumably eaten. At no time did we observe ants to attack myrmecophilous aphids; however, they carried off these species if dead or injured individuals were presented to them.

The response of the aphids to alarm pheromone (*7*) confirmed our earlier field observations that myrmecophilous species do not disperse as extensively as do nonmyrmecophilous species (Table 1). Rarely did the three aphid species that form a close bond with ants (*Aphis fabae*, *Chaitophorus viminalis*, and *C. populicola*) respond to alarm pheromone by falling from their "feeding clusters." Instead, they either walked away or waggled their bodies. The function of the nondispersive waggle is not understood.

In additional tests with *A. fabae* and *C. populicola*, we found that the dispersive response to alarm pheromone was further diminished by ant attendance. When exposed to alarm pheromone (*8*), most unattended aphids responded by walking away from "feeding clusters" (Table 2). However, aphids that had been previously tended by *F. subsericea* responded principally by waggling. The recent report (*9*) that mandibular gland secretions of *Formica fusca* can affect morph determination in *A. fabae* suggests to us that aphid alarm behavior may be altered in a similar manner. The shift in alarm response of myrmecophilous aphids from a dispersive to a nondispersive mode suggests that these aphids rely on ants for protection from predators.

In the field, we noted that *F. subsericea* responded aggressively to aphid predators. In the laboratory we placed more than 50 predacious coccinellid adults (*Ceratomegilla maculata*, *Adalia bipunctata*) on plants with ants tending *C. populicola*. Ants attacked all predators and removed them from the aphid host plant (Fig. 3). Detection of predators usually occurred only at close range (< 1.0 cm).

To determine whether alarm pheromones from injured aphids could be perceived by ants and enhance predator detection, forceps were used to carefully approach and squeeze aphids attended by ants. Ants responded to the secreted cornicle droplets by raising their antennae, opening their mandibles, turning toward the injured aphid, and attacking the forceps.

Ants were also exposed to *trans*-β-farnesene on filter paper triangles while they were tending *C. populicola*. The triangles were brought 0.5 to 1.0 cm above and to the rear of individual ants. Groups of three ants were tested 14 to 18 times with each dosage. A positive response was recorded when the ant immediately ceased antennating aphids, raised and extended its antennae forward, opened its mandibles, and walked rapidly over the aphid cluster and plant surface (Fig. 4). The ant often turned toward the pheromone source, rose up, and tried to bite the paper triangle. We observed no response to triangles treated with 0.2 ng of pheromone, but 8 of 14 groups of ants responded to triangles with 2 ng of pheromone and 13 of 18, 11 of 15, and 14 of 16 groups responded to 20, 200, and 2000 ng, respectively. Only 2 of 73 groups of ants responded to paper triangles not treated with pheromone. To be certain that ants respond to the same levels of pheromone as aphids, the same test solutions and techniques were used for two aphid species. The response threshold was 2 ng for *A. fabae* and 20 ng for *Schizaphis graminum*.

Table 2. Response of myrmecophilous aphids to alarm pheromone. Comparison is made between aphids that had been previously exposed to ants (attended) and those not previously exposed (unattended). ($N > 200$ aphids per treatment; TBF, synthetic *trans*-β-farnesene)

| Aphid species and pheromone source | Aphid response (%) | | |
|---|---|---|---|
| | Walk | Waggle | None |
| *Unattended* | | | |
| *A. fabae* | | | |
| Cornicle droplet | 94.0 | 2.8 | 3.2 |
| TBF (10 ng) | 75.8 | 20.1 | 4.0 |
| *C. populicola* | | | |
| TBF (10 ng) | 80.5 | 4.7 | 14.5 |
| *Attended* | | | |
| *A. fabae* | | | |
| Cornicle droplet | 26.3 | 71.7 | 2.0 |
| TBF (10 ng) | 23.5 | 75.1 | 1.4 |
| *C. populicola* | | | |
| TBF (10 ng) | 30.6 | 69.4 | 0 |

Formicine ants respond to an array of hydrocarbons (*10*) and may have been preadapted to respond to aphid alarm pheromone prior to the evolution of ant-aphid associations. We have found that *F. subsericea* responds to a number of hydrocarbons, including undecane, two farnesene analogs, and, to a lesser extent, citronellal.

We have demonstrated that aphid alarm pheromone plays a key role in the association between ants and aphids. Maintenance of an intact aphid aggregation is crucial to success of the association. The difference in alarm response between myrmecophilous and nonmyrmecophilous aphids supports this view. The alteration of alarm behavior of myrmecophilous aphids by ants contributes to the stabilization of the association. Persuasive evidence for the role of aphid alarm pheromone in this stabilization is the direct alarm communication between aphid and ant.

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5. These are: *Aphis fabae, Rhopalosiphum padi, Schizaphis graminum, Hyadaphis erysimi, Macrosiphum euphorbiae, Acyrthosiphon pisum, A. solani,* and *Myzus persicae* (subfamily Aphidinae); and *Chaitophorus viminalis, C. populicola,* and *Sipha kurdjumovi* (subfamily Chaitophorinae).
6. Ant colonies were maintained in soil in metal bushel baskets in a laboratory at 22° ± 2°C and 15 hours of light per day. Cotton string run through plastic tubing was used to connect ant colonies with plants bearing aphid colonies. Aphid-ant interactions were observed the first 4 days after the introduction of ants.
7. Clusters of 3 to 30 aphids were exposed to cornicle droplets produced by gently squeezing an aphid of the same species. Each species was tested 12 to 25 times.
8. Ant-attended aphids were exposed to ants for 4 days during which aphids developed from first and second instar nymphs to fourth instar nymphs and young adults. Ants were removed 15 minutes to 1 hour prior to testing aphids. Clusters of 5 to 27 aphids were tested 18 times with cornicle droplets as reported above (*7*), or with *trans*-β-farnesene in a methanol solution. Paper triangles that hold approximately 1 μl of solution were held within 1 cm of aphids for 1 minute. Aphids did not respond to other aphids that did not produce cornicle droplets or to paper triangles treated with methanol alone.
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## Lethal Interaction of Ubiquitous Insecticide Carriers with Virus


Abstract. *Large quantities of presumably nontoxic petroleum oil by-products are introduced into the environment as pesticide dispersal agents and emulsifiers. An increase in viral lethality with a concomitant influence on the liver and central nervous system occurs in young mice previously primed with such chemicals.*


Impurities or by-products which occur in pesticides and industrial compounds may cause disease in organisms that have been exposed to these agents. The toxicity or lethality (or both) associated with the herbicide 2,4,5-T (*1*), chlorinated phenols (*2*), polychlorinated biphenyls (*3*), and diphenylamine (*4*) are due to impurities introduced during manufacture or mixing of these compounds or to impurities formed during their storage or environmental breakdown. Among the pesticides known to have additives that enhance their toxicity are aldrin (*5*), chlordane *p,p'*-DDD, diazinon, and trichlorofon (*6*). Contamination of formulations by nonbiodegradable persistent material is a current and ongoing problem with an impact on animal and human health (*7*). For example, a group of Canadian children living within the area of a forest sprayed with fenitrothion and DDT suffered a particular combination of central nervous system and liver pathology after they had been exposed to a virus. We observed that when young mice were first exposed to certain combinations of the above-mentioned insecticides and then infected with a nonlethal dose of mouse encephalomyocarditis (EMC) virus, the effect of the virus was enhanced (*8*). The insecticide used in the forest spray area was applied as an emulsified concentrate consisting of toxicant, one or more solvents, and a blend of emulsifiers. The solvents were used as diluents and together with emulsifiers are useful in the even dispersal of the insecticide particles. Emulsifiers also act as wetting agents on foliage (*9*). The type of formulation in which a pesticide is dispersed can significantly affect the toxicity hazard (*10*).

Little work has been done on insecticide carriers, and toxicological data are almost nonexistent. These carriers are usually considered nontoxic and therefore find wide use in industrial and domestic products. In addition they have received very little attention from environmental research groups or agencies. Consequently, we examined the toxicity of the particular solvent carriers in the insecticides. Our study shows that the emulsifiers and solvents are, in fact, active in vivo chemicals which influence viral lethality.

Swiss white mice (Biobreeding, Ottawa) from an outbred strain (ICR) were mated in our own laboratories and allowed to deliver their young (884 mice). Contact of the newborn mice with the chemical under test was started at 24 hours of age. All groups of mice were studied simultaneously. To avoid inbreeding, we purchased new females for each group of experiments.

Purified fenitrothion and DDT were obtained from commercial sources and their purity was confirmed by gas chromatographic analysis (> 99 percent).

Fenitrothion was prepared in a solvent (Aerotex 3470, Texaco of Canada) with two emulsifiers (Toximul MP8, Chas. Tennant & Co. of Canada Ltd., Toronto, Ontario; Atlox 3409, Atlas Chemical Industry Ltd., Brantford, Ontario). Samples of each emulsifier and solvent (as used) were obtained from the suppliers. Thin-layer chromatographic analysis of the solvent and the emulsifiers gave several bands; subsequent examination of these fractions by gas chromatography indicated that several chemical components were present in each band. The ultraviolet absorption spectra of the solvent and emulsifiers showed maxima at 274, 265, and 255 nm; these are typical values exhibited by alkylated aromatic compounds (*11*). Corn oil was used as a control carrier because of its nontoxic properties and solubility characteristics.

Dosages of insecticides were based on established median lethal doses ($LD_{50}$) (*12*) and on our own trials in which the toxicity of various dosages was determined relative to the age of the animals (DDT, 124 mg per kilogram body weight and fenitrothion, 7.6 percent, by volume). The emulsifiers and solvent were mixed with corn oil (7 : 1000); this is the ratio for the carrier of fenitrothion used in this commercial spray formulation. Each solution was applied with a small camel-hair brush to the abdomens of the young animals. Solutions were painted once daily from 24 hours after birth (day 1) until day 11. The following solutions in corn oil base were applied: (i) corn oil alone; (ii) pure DDT; (iii) commercial fenitrothion (which contains the emulsifiers and the solvent); (iv) DDT plus the commercial fenitrothion; (v) DDT plus 3.8 percent pure fenitrothion (equivalent to DDT plus commercial fenitrothion without emulsifiers and solvent); and (vi) emulsifiers and solvent.

Stocks of EMC virus, originally obtained from the National Institute for Medical Research, Mill Hill, London, and supplied as the primary mouse-kidney cell yield, were used. The titer of the stock virus was between $10^6$ and $10^7$ median tissue culture infectious doses ($TCID_{50}$) per milliliter. The animals were observed for the development of symptoms.

Most deaths occurred within 5 days of viral injection. Long-term survivors were observed until day 23, and then were killed by cervical dislocation. Specimens of liver and brain were obtained, fixed in formalin, stained by hematoxylin and eosin, and observed by light microscopy. Other matched liver specimens were frozen and stained by oil-red-O to visualize localization of lipids. Additional tissue was obtained from the affected animals at the time of death for viral and