Attraction of the Parasitic Mite *Varroa* to the Drone Larvae of Honey Bees by Simple Aliphatic Esters

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An important parasitic threat to honey bees, the mite *Varroa jacobsoni*, is attracted to its major prey, drone larvae, by methyl and ethyl esters of straight-chain fatty acids, in particular methyl palmitate. These esters were extracted from drone larvae with *n*-hexane and were identified by gas chromatography–mass spectrometry. Their behavioral effect was evaluated with the use of a four-arm airflow olfactometer.

THE ACARIAN *Varroa jacobsoni* OUDEMANS is the most serious threat to the honey bee *Apis mellifera* (*1, 2*). It clings to the bees and sucks their hemolymph, thus shortening their life, causing various physical, functional, and behavioral anomalies, as well as favoring secondary infections, in particular viroses. Hundreds of thousands of colonies have been lost in Europe and in Asia; the mite was first found in the United States in September 1987. The severe damage this mite causes to entire colonies is of importance not only for the honey industry but also in the pollination of many important crops. At the present time, acaricides keep *Varroa* partially under control, but their use in beehives is questionable in that their useful effects are short-lived and their use has harmful side effects (*3*). Furthermore, the *Varroa* females are physically protected during the period they spend in the sealed cells. Adult female *Varroa* live on adult worker and drone honey bees, which mostly act as intermediate hosts and as means of intercolony transmission. The mites begin their reproductive cycle by entering the cells of bee larvae. They leave the adult worker bees and are attracted by larvae (mostly by those of drones) about 2 days before their cells are capped (*4*). In the cells of drone larvae, the mite finds more favorable conditions for its proliferation: male bees require a longer time for their development, which allows larger numbers of female mites to reach maturity, and the average temperature of male cells lies nearer the optimal growth temperature of *Varroa* than that of the workers' cells (*5*).

The attraction of mites suggests that bee larvae produce a signal, and the frequent occurrence among invertebrates of chemical signals made it probable that, in this case also, a chemical attractant directs the parasite toward its prey. Such an interspecific chemical signal, beneficial to the receiving organism, is called a kairomone. We have isolated and identified ten straight-chain fatty acid esters from the extract of bee larvae, some of which efficiently attract *Varroa* females at the normal temperature of the hive. This finding could become the basis of new approaches in the control of *Varroa*.

The study of airborne semiochemicals in the host-finding process of *Varroa* made use of a plexiglass four-arm airflow olfactometer previously described (*6, 7*) and adapted for this study (dimensions: exposure chamber, 56 mm by 56 mm by 2 mm; inside diameter of the arms, 2 mm). Four odor fields were created in the star-shaped exposure chamber by sucking air out through the hole in the center of its floor. Each of the fields of the olfactometer was thus swept gently by an air flow (0.9 ± 0.1 liter per hour) from the corners toward the center. All the experiments were run at 32° ± 1°C and at a relative humidity of 80 ± 5%, that is, under the usual conditions normally present in the beehive and found optimal for the response of the mite (*5*). *Varroa* females, which had been taken from a colony and maintained on worker bees at 22°C for 5 to 7 days in a breeding cage, were individually placed at the center. The mites could walk freely in the four fields and their positions were recorded on a grid of 5 mm by 5 mm squares every 5 s for a total of 375 s (in 5 s, a mite moves approximately one square). Seventy-five positions were noted per individual, and 56 mites were used sequentially for each test so that 4200 positions were noted in each experiment. The results were evaluated in two complementary ways: (i) the distribution of *Varroa* can be illustrated by increasing levels of tint, from white to black, to represent their cumulated presence in each field during the time of the experiment with the use of a computer program (*8*) (Fig. 1); and (ii) the relative time spent by the mites in the odorized and in the control fields has been evaluated by noting the ratio of the number of the positions of the mites in each field to the total number of observations (4200, Table 1) and plotted these values as a function of time (Fig. 2). For the statistical analysis, we calculated the relative number of mites in the odorized and control fields by multiplying the ratio of units present in the two types of fields by the total number of mites tested (56). We then checked the statistical significance of this proportion with a χ^2 test against a random distribution.

The mites first move in the central zone, which they explore for a variable amount of

Table 1. Responses of *Varroa* females at 32°C as a percentage of time spent by mites in the olfactometer fields (cumulative results for the 75 time periods). In all cases, the mites spend a greater amount of time in the central area than would be expected, as this area is not smooth but is drilled to permit the aspiration of air. Abbreviations: DL, live drone larvae; EDL, extract of drone larvae; F2, the behaviorally active fraction; MP, methyl palmitate; EP, ethyl palmitate; ML, methyl linoleate; MIX, a mixture of these three components; and MP-22, MP at 22°C. Results are also shown for a control experiment in which all four fields were unodorized.

Compound	Area		
	Center	Control	Odorized
DL	28	20	52
EDL	40	16	44
F2	47	13	39
MP	20	19	61
EP	42	13	45
ML	35	22	43
MIX	21	27	52
MP-22	46	22	32
Control	41	30	29

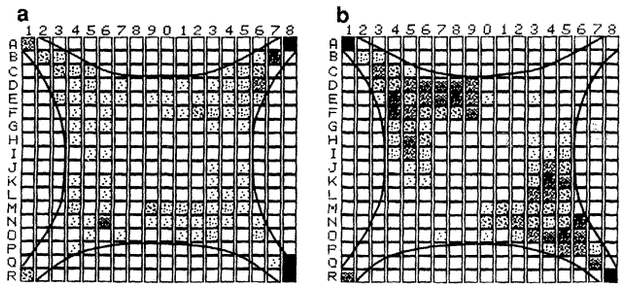

Fig. 1. Spatial distribution of the *Varroa* females in the olfactometer. Values were grouped into eight classes of density characterized by increasing levels of tint distributed from white to black (white: 0 to 5 points; black: >35 points). (**a**) In the control experiments, the four fields were odorless. (**b**) Response to drone larvae odor. Two of the four fields (upper left and lower right) were odorized with 15 live drone larvae, whereas the two other fields were odorless.

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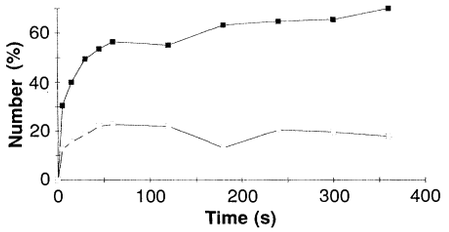

Fig. 2. Distribution of the *Varroa* females in the olfactometer as a function of time. (Closed squares: odorized fields (methyl palmitate); open squares: control fields.

time. They then move into the odorized or into the control arms. Some individuals remain within one arm during the entire experiment; others explore the four arms. The mite is not visible while in the central area (corresponding to 36 squares) because of the airflow extraction device and is simply reported as being in the central area.

The random distribution of the mites in the absence of any stimulus is shown in Fig. 1a: clean air was allowed to flow from the four corners toward the center. For the tests, the air flowing from two diagonally opposed arms passed first through glass flasks containing the odor to be tested (*9*). Fifteen live drone larvae (8 days old) were placed to the upper left and lower right airflows toward which the *Varroa* mites were attracted (Fig. 1b); this response suggested the existence of a kairomone ($\chi^2 = 7.02$, $P < 0.01$). This kairomone is soluble in hexane (*10*). The total hexane extract is very attractive ($\chi^2 = 7.76$, $P < 0.01$). We fractionated 8 ml of this extract [equivalent to 48 larvae (48 lar-eq)] by chromatography on a column of silica gel (2.5 g). Three fractions were collected: F_1 was eluted with 4 ml of hexane, F_2 with 4 ml of dichloromethane, and F_3 with 4 ml of ethyl acetate. These eluates were evaporated to dryness in a stream of dry nitrogen, redissolved in hexane (2.7 ml), and tested. Fractions F_1 and F_3 were totally inactive, whereas fraction F_2 was quite attractive, at the level of 1.2 lar-eq ($\chi^2 = 7.28$, $P < 0.01$, Table 1). Fraction F_2 was analyzed by gas chromatography–mass spectrometry (Finnegan-MAT INCOS-50), and the resulting chromatogram showed ten peaks that were identified by comparison with authentic samples (Fig. 3).

Each of these substances (commercial samples, from Sigma) was then used in a bioassay at the level of 1 μg (4 to 100 lar-eq). Methyl palmitate (MP) gave a very strong attractive response ($\chi^2 = 12.46$, $P < 0.001$) whereas ethyl palmitate (EP) and methyl linolenate (ML) induced weaker responses ($\chi^2 = 9.59$, $P < 0.01$; and $\chi^2 = 3.95$, $P < 0.05$). The other components did not elicit any significant attraction. The mixture (MIX) of MP (1 μg), EP (0.35 μg), and ML (2.27 μg) in their naturally occurring ratio was also attractive ($\chi^2 = 4.27$, $P < 0.05$).

We then tested two odors at the same time through adjacent arms to providing complex preference test situations. The sample was as attractive as the fraction F_2 [$\chi^2 = 0.19$, not significant (NS)], which suggested that the seven other components of this fraction do not exert a synergistic effect on the three active ones. The attractiveness of the three active components was further tested in mixtures; the order of attractiveness was MP > EP > ML (MP versus EP, $\chi^2 = 0.61$, NS; MP versus ML, $\chi^2 = 5.05$, $P < 0.05$; and EP versus ML, $\chi^2 = 1.39$, NS). The sample was not more active than MP alone ($\chi^2 = 0.10$, NS).

In the presence of methyl palmitate, the behavior of *Varroa* was a typical oriented attraction: there was no significant difference between arrest times (more than ten consecutive seconds in the same place) in the odorized fields (35%) and the odorless fields (42%). Attraction of the mites by this component was very strong and quickly evident: 50% had reached the odorized fields in 30 s (Fig. 2).

As the crucial role of the temperature in the *Varroa*-bee relationships had already been demonstrated (*11*), we checked the influence of temperature on the effect of the attractive components. For example, the most active component, methyl palmitate, which was highly attractive at 32° ± 1°C, was totally inactive at 22°C (Table 1), which is in agreement with the optimal temperature of the biological interaction.

The hexane extract of 5-day-old worker larvae was equally attractive to *Varroa* ($\chi^2 = 3.96$, $P < 0.05$). However, when given the choice in the olfactometer between live worker and male larvae, the acarian selectively and significantly ($\chi^2 = 4.44$, $P < 0.05$) chose the latter. The hexane extract of worker larvae contained the same three active components (MP, EP, and ML) as the drone larvae, but in smaller amounts.

The simple aliphatic methyl and ethyl esters isolated from bee larvae do not appear to have been identified previously from natural sources, but this may be due to the frequent use of alkaline hydrolysis in the first steps of the study of lipids. The identification of such simple substances as baits for *Varroa* mites should make it possible to devise a number of new techniques to eradicate these pests. The acarians would be first attracted, then trapped in the beehives by these compounds. Putting our findings into practical use, the beekeepers may have at hand a diagnostic method and also a good way to treat their beehives and fight *Varroa* with natural products that do not appear to be harmful to bees.

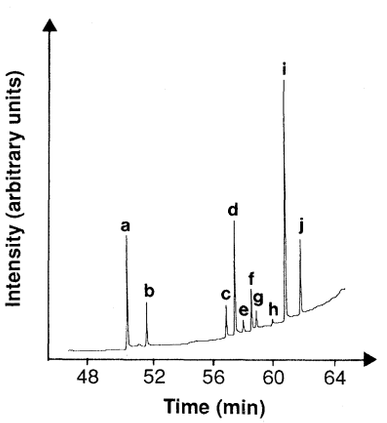

Fig. 3. Chromatogram of the behaviorally active fraction F2. Gas chromatograph: Carlo Erba GC 6000; column: CW 20 M, 50 m, inner diameter 0.32 mm. Amount expressed per larvae equivalent: (a) methyl palmitate, 0.26 μg; (b) ethyl palmitate, 0.09 μg; (c) methyl oleate, 0.07 μg; (d) methyl stearate, 0.26 μg; (e) ethyl oleate, 0.03 μg; (f) ethyl stearate, 0.08 μg; (g) methyl linoleate, 0.05 μg; (h) ethyl linoleate, 0.01 μg; (i) methyl linolenate, 0.59 μg; and (j) ethyl linolenate, 0.18 μg.

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9. After two individual trials, the entire olfactometer was rinsed with ethanol and with distilled water and then dried. New runs were resumed with a new batch of the test material. After seven such double runs, the entire apparatus was turned 90°. The complete experiment involved four right-angle turns (thus requiring $2 \times 7 \times 4 = 56$ individual *Varroa* mites). Such a complex procedure was designed to eliminate any potential bias, such as light gradients, traces of semiochemicals, influence of the orientation, presence of trail pheromones, and so forth.
10. We collected 300 drone larvae 2 days before their cells were capped. The larvae were placed in 50 ml of hexane for 1 hour at 20°C; the resulting extract (6 larvae-equivalents per milliliter = 6 lar-eq/ml) was tested by evaporating 0.2 ml of this solution (1.2 lar-eq) on a 2 cm by 2 cm glass microfiber filter (Whatman GF/F) onto a glass flask through which passed the diagonal airflows. The other flasks received a control filter with the solvent alone.
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