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# CUTICLE ALKANES OF HONEYBEE LARVAE MEDIATE ARRESTMENT OF BEE PARASITE *Varroa jacobsoni*

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**Abstract**—The ectoparasitic mite *Varroa jacobsoni* invades worker brood cells of the honeybee *Apis mellifera* during the last 20 hr before the cells are sealed with a wax cap. Cuticle extracts of 8-day-old worker honeybee larvae occupying such brood cells have an arrestment effect on the mite. The mites run for prolonged periods on the extract, systematically returning onto the stimulus after touching the borders of the treated area. Mites increase walking speed and path straightness in response to increasing doses of a nonpolar fraction of the cuticle extract. Saturated straight-chain odd-numbered $C_{19}$—$C_{29}$ hydrocarbons were identified by thin-layer argentation chromatography and gas chromatography–mass spectrometry as the most active constituents, with branched alkanes also contributing to the arrestment effect of this active fraction. Analysis of the behavior responses to synthetic *n*-alkanes indicate that the response is probably based on a synergism between the different alkane components of the fraction rather than to an individual compound.

**Key Words**—*Varroa jacobsoni*, Acari, Varroidae, mite, *Apis mellifera*, Hymenoptera, Apidae, honeybee, chemoreception, host selection, cuticle, hydrocarbons, alkanes.

## INTRODUCTION

The ectoparasitic mite *Varroa jacobsoni* Oud. (Acari, Varroidae) threatens colonies of honeybees *Apis mellifera* L. worldwide. It enters brood cells of male

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honeybee larvae 20–40 hr and cells of female worker larvae 0–20 hr before operculation (Boot et al., 1992). Worker bees seal the cells of 9-day-old worker and 10-day-old drone larvae with a wax cap (Winston, 1987). The mite reproduces during host development within the brood cell. On emergence of the young bee, mother and daughter mites leave the brood cell and the males die. Being ectoparasitic, the mites feed on host hemolymph.

The short time span of 20 hr during which the mites invade brood cells suggests recognition of hosts of the appropriate age by *Varroa*. Indeed. three fatty acid esters (methyl palmitate, ethyl palmitate, and methyl linolenate) identified from 9-day-old drone larvae proved attractive to *Varroa* in an olfactometer (LeConte et al., 1989). In addition, palmitic acid, the probable precursor of methyl palmitate, is present in the headspace over 8-day-old worker larvae and attracts the mites, eliciting an upwind walking response when presented in an airstream (Rickli et al., 1992). These products may therefore serve as host-finding cues. After operculation, the bee larva spins a cocoon. During this period, the mite is highly mobile but shows a strong attachment for the free surface of the larva (Donzé and Guerin, 1994). Two functions of this behavior appear plausible: (1) to avoid being crushed between the larval body and cell wall during cocoon spinning and (2) to avoid being excluded from the larva by the newly spun cocoon on the cell wall.

We have observed that mites show an arrestment response on a substrate treated with cuticle extract of 8-day-old worker larvae in vitro. Mites walking on the area treated with this extract systematically change their walking direction upon touching the border of the extract to return to the treated area (see Figure 1 below). In this study, we identify chemostimuli mediating this arrestment response of *Varroa*. Furthermore, an analysis of tracks made by *Varroa* on the treated area describes major features of the mite's response to the cuticle extract and one of its components.

## METHODS AND MATERIALS

*Use of Abbreviations:* straight-chain alkanes are abbreviated as *n*-$C_x$ where *x* indicates the number of carbon (C) atoms followed by the number of double bonds. *br*-C*x* stands for internally branched alkanes, most of them monomethyl alkanes, but also including some dimethyl alkanes. Thus *br*-$C_{25:0}$ signifies a chain of 25 C atoms with either one or two methyl groups at an unspecified location within the chain, the molecule having no double bonds.

*Mites.* Lots of 60–100 *Varroa* visible on the surface of adult bees in heavily infested bee colonies were collected and held for two to seven days on their hosts in the laboratory before bioassay. Some 15–30 min before a test, a group of 10–15 mites was removed from the bees and held in a humidified glass tube

at room temperature (18–21°C) until a test run. Each test solution was assayed with mites of at least two different lots on different days. Tests were conducted with batches of test solutions all assayed on the same day and accompanied by at least one solvent control. The daily sequence of solutions was randomized. Prior to tests of fractions of bee cuticle extract and dilution series, some mites were subjected to negative (solvent) and positive (active fraction of extract) controls to ascertain the responsiveness of the lot of mites being employed in the bioassays.

*Cuticle Extract and Fractionation.* Eight-day-old *A. mellifera* worker larvae were extracted using their size and weight (over 120 mg) (Thrasyvoulou and Benton, 1982) as an index of age. These larvae were chosen because of the relative ease of access to them before operculation. Fifty to 200 larvae were submerged for 15 min in 10 ml *n*-hexane (Merck, analytical grade) and the resulting extract concentrated to 1 ml under a gentle flow of nitrogen. All extracts were stored at −20°C. Thin-layer chromatography (TLC) plates (Merck, ready-made Silica Gel 60 analytical plates) were conditioned by running them twice in a mixture of methanol—chloroform 1:2 and drying. After loading 0.2–1.0 ml of extract (20–100 larva equivalents, leq), the plates were fully developed in hexane followed by toluene, and to two thirds in hexane—diethylether—acetic acid 70:30:1. A strip was cut off the plate and developed by charring with 50% $H_2SO_4$ and heating to 140°C until bands were visible. Bands corresponding to the fractions thus visualized were then scraped from the rest of the plates and extracted with dichloromethane ($CH_2Cl_2$). Fractions were concentrated under $N_2$ to a volume equivalent to that of the extract and then tested at a level corresponding to 12 leq on the test arena. The most apolar fraction, F1, was further separated into complex wax esters and front-running hydrocarbons (HC) by separation of F1 alone in hexane on the same TLC plates. This HC fraction (F1A) was found active and tested in amounts ranging from 0.6 to 12 leq.

To distinguish between saturated and unsaturated alkanes present in fractions F1 and F1A (above), it was necessary to separate saturated HCs from the rest. For this, cuticle extract was separated in hexane on TLC plates impregnated with silver nitrate (Aitzetmüller and Guaraldo Goncalves, 1990) where only *n*- and *br*-alkanes migrated. The nonmigrating fraction of more polar products was bioassayed as such. In addition, alkenes and alkadienes were purified from this more polar material by elution in hexane on ready-made TLC plates and tested as "alkenes." The silver nitrate-developed fraction containing the saturated alkanes was further separated into straight-chain and branched alkanes by adsorption of the straight-chain products onto a molecular sieve of zeolith $CaAlSi_3O_8$ (O'Connor et al., 1962). After conditioning the sieve (Merck 5705, pore size 0.5 nm) at 260°C under vacuum (0.6 mm Hg) for 4 hr, 1 g was added to the sample of saturated compounds dissolved in 2–3 ml isooctane and refluxed at 110–115°C for 8 hr. The nonadsorbed *br*-alkanes were recovered from the

isooctane by concentration under $N_2$. Recovery is never complete, and in this case some *br*-alkanes were also adsorbed. The initial amount of HCs exposed to the molecular sieve was 50–70 leq, the amount bioassayed was 16 leq *br*-alkanes (12 $\mu g/cm^2$).

*Identification and Quantification.* Gas chromatography–mass spectrometry (GC-MS) was employed to identify constituents of active TLC fractions. Samples were injected on-column onto a 15-m BGB (Zürich) high-temperature/high-resolution fused silica capillary column in a Hewlett Packard 5890 Series II gas chromatograph coupled to a HP 5971A mass-selective detector. The nonpolar column (5% phenyl, 95% methylpolysiloxane, 0.25 mm ID, and 0.12-$\mu$m film thickness) was temperature programmed either from 60°C after 2 min (He with 16 kPa head pressure, constant flow) or from 100°C after 2 min (He at 28 kPa, constant flow) at 10°/min to 370°C. The mass selective detector (190°C) was set to a scan range of $m/z$ 50–650. Retention times and mass spectra of unknowns were compared with those of authentic samples.

Gas chromatography with flame ionization detection (GC-FID) allowed us to estimate accurately the quantities of the compounds present in the different fractions. For this a Carlo-Erba HRGC 5300 (Mega Series) equipped with a 30-m nonpolar high-resolution fused silica capacity DB-5 column (J&W, California: 0.32 mm ID and, 1-$\mu$m film thickness) was employed with splitless injection at 240°C and the FID detector at 300°C. The column was temperature programmed for a fast analysis from 200°C after 2 min at 5°/min to 340°C and held for 16 min or, for more detailed analysis, from 60°C after 2 min at 5°/min to 300°C and held for 80 min with He at a flow rate of 1.35 ml/min. Quantification of compounds identified by GC-MS was made by analysis of five separate extracts of 100 larvae held for 15 min in hexane and concentrated to 1 ml. Of each extract, 0.1 leq with 50 ppm of $n$-$C_{24:0}$ as internal standard were injected. In this study, only components making up more than 1% of the cuticular extract were quantified.

*Bioassay and Data Analysis.* Three concentric circles of 12, 24, and 36 mm diameter were drawn with a fine 0.1-mm ink pen of the underside of a semipermeable biological membrane (Baudruche, John Long Inc.) (Figure 1). This membrane was then washed twice in hexane and once in acetone and stretched over a small water bath providing a humidity of $\geq$90% and a temperature of 32°C on the surface of the membrane. Some 50–200 $\mu$l of the test solution were spread with a 10-$\mu$l micropipet on the band between the inner and middle circles called the treated area. The surface of the treated area (3.4 $cm^2$) made up roughly 1.7 times the surface of an 8-day-old larva (1.8–2.4 $cm^2$). A mite was deposited with a fine brush in the center of the area. After 300 sec or upon leaving the outer circle, which ever came first, the test was terminated and the mite removed.

Synthetic saturated and monounsaturated odd-numbered $n$-$C_{19}$ to $n$-$C_{29}$

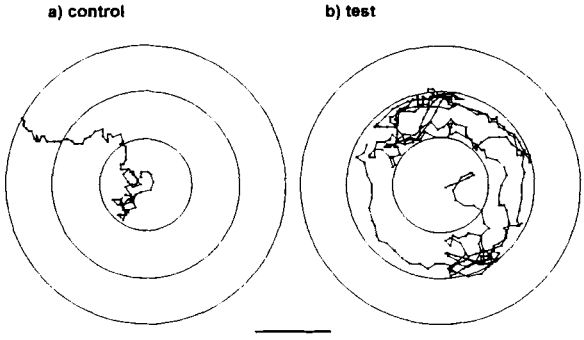


FIG. 1. *Varroa* tracks on a water-permeable biological membrane with test material applied between the inner and middle circles: (a) solvent alone (control) and (b) 30 $\mu$g $n$-$C_{21:0}$/cm$^2$ (test). Mites were released in the center and their paths drawn for the first 60 sec of the 5 min test or until the mite left the outer circle, which ever came first. On solvent control, the animal moved without return through the treated area in 5 sec. Once on the area treated with $n$-$C_{21:0}$ (b) the mite moved for 147 sec on it, returning 61 times to the stimulus after contacting the border. Bar represents 10 mm.

($\geq$97% purity by GC, Sigma) were dissolved in hexane and tested at doses of 30 $\mu$g/cm$^2$ initially. Binary and ternary combinations of compounds of neighboring chain lengths were also tested at the same total amount. This quantity was visually similar to treating the membrane surface of the test arena with 12 leq of fraction F1. Additionally, doses of $n$-$C_{21:0}$ ranging from 1.5 to 15 $\mu$g/cm$^2$ were tested. A solution of the synthetic $n$-alkanes $C_{21}$, $C_{23}$, $C_{25}$, $C_{27}$, $C_{29}$ at proportions (0.2:3:7:12:5) close to that found in cuticular extracts was tested at doses from 3 to 30 $\mu$g/cm$^2$ of treated area, corresponding to 2–24 leq $n$-alkanes. To test for any purely physical effect of the HCs on the arena surface, a comparison was made with Vaseline at 15 $\mu$g/cm$^2$ applied to the same surface. Two attractants for *Varroa*, i.e., palmitic acid (Rickli et al., 1992) and methyl palmitate (LeConte et al., 1989) were tested at doses of 3 and 1.5 $\mu$g/cm$^2$, respectively.

Test runs were recorded on VHS video from perpendicularly above the membrane, and the walking behavior was subsequently analyzed using a computer program linking the video observations with time (The Observer, Noldus Information Technology). In this manner, the time spent by a mite on the different zones of the arena and walks and stops were quantified. Once on the treated area: (1) the number of contacts made by the mite with the borders of the treated area, (2) returns at the border back towards the treated area, and (3) moves onto the untreated surface were quantified. The results from 671 runs on the solvent control were pooled and used to define a standard behavior for three

parameters of the walks, which showed highly asymmetric value distributions. In all, 95% of the mites in these controls: (1) moved for less than 41.0 sec on the test band (indexing for a general activity of the animal), (2) showed less than 2.34 returns/10 sec when moving on the test band (by contrast with chemostimulated mites that returned from test band borders much more often in the same time span), or (3) made fewer than four returns per run (border recognition). If an animal showed a value above any of the three 95% limits in a test situation, it was considered to react towards the solution applied. The number of runs with values above at least one of the 95% limits on a given test solution was compared to the number recorded for the other solutions tested on the same day with the Fisher exact test. A double check showed that none of the control groups was significantly different from the pool of control runs. This ruled out that relative activities of test solutions could be falsely judged considering the large day-to-day variations in the mites' walking behavior.

Additionally, the total time spent by the mites in contact with a solution (divided into moving and stopping periods) was analyzed. The contact time of each mite was plotted in notched box plots (McGill et al., 1978). If the 95% confidence intervals of the medians did not overlap between test and control, we considered the result significant. The notched box plot results are only stated if they differed from the results obtained by the method of 95% limits for the other parameters described above.

*Analysis of Tracks.* For this part of the study we made 10 tests each on stimulus doses corresponding to 0 (solvent control), 0.6, 1.2, 2.9, and 5.9 leq of the active nonpolar TLC fraction after removal of the wax esters (i.e., fraction F1A). All test runs on fraction F1A were done on the same day, i.e., with mites of the same lot. Tracks made by mites of a separate lot on $n$-$C_{21:0}$ at 30 $\mu g/cm^2$ (Figure 1) and on the corresponding solvent control ($N = 10$ mites per treatment) were also analyzed. Tests chosen for examination of *Varroa* tracks are representative of the mite's behavior to chemostimuli as observed using the bioassay system described above.

Track coordinates were obtained from an $x,y$ grid laid on the video screen. A clock fed into the video image during recording permitted determination of frame number per second, which in 80% was 25 (video norm) and in 20% was 26 frames. Distances in $x,y$ units were registered at a resolution of 0.34 mm (length of the mite 1 mm). For this part of the study, the position of the animal's pedipalps was noted every fifth frame, i.e., every 0.2 sec for the first minute of the run. *Varroa* have been suggested to bear chemosensitive sensilla on the tarsi of leg pair 1 (P1) (Ramm and Böckeler, 1989; Milani and Nanelli, 1988) and the pedipalps (Liu, 1990). For each $x,y$ coordinate, an index was added to the records indicating whether the mite was fully on the treated area with all chemosensitive sites, in contact with the ink circle (i.e., on the border of the treated area), or outside the treated area. Statistical analysis was performed using

a post hoc ANOVA (Tukey HSD test) on distances covered and on angular data (see below) resulting in a matrix of pairwise comparisons between the treatments.

The analysis applied here was made on track segments where the mites walked fully on the treated area. (Tracks from the release point in the center of the arena to first contact with the stimulus, tracks after leaving the extract, and tracks made on the borders of the treated area are not dealt with because they reflect quite different situations for the mites.) The raw $x,y$ coordinates with the origin ($x = 0$; $y = 0$) in the center of the arena were computerized and converted into real $x,y$ distances in millimeters. The angle of each $x,y$ pair to the 0° axis (the $x$ axis here) of the coordinate system was calculated [$\arctan(y/x) * 180/\pi$; angles for $y$ or $x = 0$ defined as 0°, ±90°, or ±180°]. With the formula $(\Delta x^2 + \Delta y^2)^{0.5}$, the distance covered was calculated, and with the formula $\arctan(\Delta y/\Delta x) * 180/\pi$ (angles for $\Delta x$ or $\Delta y = 0$ defined as above) the direction moved per 0.2-sec interval (vector) was obtained. The mite's turn angle was calculated as the difference in displacement direction between two subsequent 0.2-sec vectors. However, the distribution of turn angles showed a bias for 90° and subdivisions thereof, which is an indication that the distance traveled by the mites (mean speed on solvent control for fraction F1A of 2.85 mm/sec) in the sampling interval chosen was inadequate to properly characterize the true angles described. Since *Varroa* is very quick in reacting to loss of stimulus at the border of the treated area (duration of one border contact followed by a return observed in one frame was 0.04 sec), we still decided to sample at 0.2-sec intervals rather than at longer ones. However, we calculated a running mean of the turn angles over triads of consecutive vectors, and natural logs of these means were used in the general path description. Duration and frequency of stops were not dealt with because mites walked for prolonged periods on the areas treated with 1.2, 2.9, 5.9 leq F1A and with 30 $\mu$g $n$-$C_{21:0}$/cm$^2$. Stops might therefore be due to exhaustion, something which has nothing to do with chemoreception.

We further broke the paths into segments between returns from the border onto the treated area to the next border contact. For these segments we calculated the walking speed (in millimeters per second) and angular velocity, i.e., the summed absolute turn angles per second. Statistical analysis was carried out on speed and angular velocity using the Tukey HSD test.

## RESULTS

*Behavior Responses to Cuticle Extract.* The responses of the mites were tested to eight visible fractions including the nonmigrating material on the application band of the TLC plate employed to separate the cuticle extract. In addition, a stripe called F2, which revealed no material on charring between the

front-running fraction F1 (most apolar) and F3, was tested as the blank control. The mites only showed a response to TLC fraction F1, containing HCs and wax esters, to the same extent as they did to the cuticle extract (Table 1). A response was observed on the TLC fraction F5, containing fatty acid methyl and ethyl esters, in terms of increased duration of stops on the treated area from the outset of tests (notched box plots). The fraction containing the free fatty acids (F7) elicited a response no different to controls. After separating the active fraction F1 further into complex wax esters and simple HCs (fraction F1A), the latter was also as active as the total cuticle extract or F1. *Varroa*'s response to 0.6 leq of fraction F1A was significantly different than that shown to the solvent control (21% vs. 5%, respectively; $P < 0.05$) and on higher doses of F1A the difference to the control was more pronounced (33%, 50%, 49%, and 70% of

TABLE 1. RESPONSES OF *Varroa* TO WORKER HONEYBEE LARVA CUTICLE EXTRACT AND FRACTIONS THEREOF[a]

| Extract tested | Amount (leq) applied/cm$^2$ | No. Mites tested ($N$) | Runs ($N$) over at least one limit[b] | Mites reacting (%)[c] |
|---|---|---|---|---|
| Control | | 49 | 9 | 18a |
| Cutice extract | 12 | 47 | 33 | 70b |
| TLC fractions | | | | |
| Control | | 224 | 25 | 11a |
| F1 (apolar) | 12 | 41 | 30 | 73b |
| F2 to F9 | 12 | 19-31 | 5[d] | 16[d]a |
| $AgNO_3$ | | | | |
| Control | | 50 | 3 | 6a |
| Saturated HC's | 12 | 30 | 15 | 50b |
| Alkenes | 12 | 30 | 4 | 13a |
| Polar compounds | 12 | 30 | 3 | 10a |
| Molecular sieve | | | | |
| Control | | 82 | 6 | 7a |
| Saturated HC's | 6 | 35 | 23 | 66b |
| Branched alkanes | 16 | 29 | 5 | 17a |

[a] For further explanation see text. TLC: thin-layer chromatography separation of extract into front running fraction (F1) and eight other bands (F2-F9). $AgNO_3$: Argentation TLC separation of saturated hydrocarbons (HC) from more polar constituents of the cuticle extract. Molecular sieve refers to separation (by adsorption) of straight-chain HCs from branched ones in the saturated HC fraction of the cuticle extract obtained by argentation TLC. Six larva equivalents (leq) of the saturated HCs weigh 12 $\mu$g whereas 16 leq of the branched alkanes are required to contain as much.

[b] See bioassay and data analysis section of Methods and Materials.

[c] Within test groups, percentages followed by different letters are significantly different at $P < 0.05$ (Fisher exact).

[d] Highest values obtained per TLC fraction F2-F9.

the mites responded to 1.2, 2.9, 5.9, and to 12 leq, respectively, $N > 21$). The wax esters were not active. Alkanes proved active, whereas alkenes (and alkadienes in the same fraction), on their own or together with all other more polar compounds such as fatty acids, fatty acid esters, or alcohols, were inactive in our bioassay.

The saturated HCs obtained by argentation TLC were further purified by removing *n*-alkanes with a molecular sieve. *br*-Alkanes remaining in solution were not active as judged by the absence of typical returning behavior at the borders of the treated area. However, the time the mites spent moving on the area treated with *br*-alkanes increased vis-à-vis the control (notched box plots).

*Chemical Analysis of Cuticle Extract.* GC-MS analysis showed that fraction F1, the only active fraction from the TLC analysis of cuticle extract (Table 1), contained *n*-alkanes, *br*-alkanes, alkenes (alkadienes only in minor quantities) and wax esters composed of $C_{16:0}$ or $C_{18:1}$ fatty acids esterified with even-numbered $C_{24}$ to $C_{32}$ alcohols. These wax esters were removed by running F1 again on an analytical TLC plate in hexane alone. Fraction F5 of the cuticle extract was shown by GC-MS to be composed mainly of nonanal, $C_{12}$–$C_{18}$ even-numbered fatty acid methyl and ethyl esters, and to a lesser extent the corresponding fatty acids, possibly arising from the former via hydrolysis on the TLC-plates. Free $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids and $C_{24}$–$C_{30}$ even-numbered alcohols were identified in fraction F7.

GC-MS indicated that separation of cuticle extract by argentation TLC with hexane provided a front-running fraction that contained alkanes only, while the other constituents of the extract did not migrate. The branched HCs were harvested from this saturated HC fraction by using a molecular sieve that took up the *n*-alkanes to almost 100%.

Estimation by GC-FID of quantities of different types of HCs indicated that cuticle extract contains 2.67 $\mu$g hexane-soluble material/cm$^2$ (SD $\pm$0.40 $\mu$g) of larva. The extract was made up of 75% saturated and 25% unsaturated HCs (Figure 2). *n*-Alkanes were found to make up 64% of the saturated material and *br*-alkanes 36%. Heneicosane (*n*-$C_{21:0}$), active on its own, was present in all extracts at amounts of $\leq$1% of cuticle extract or $\leq$ 0.03 $\mu$g/cm$^2$ of larva. Even-numbered *n*-alkanes also were present but at less than 1%.

*Behavior Responses to Synthetics.* A response by *Varroa* similar to that observed on cuticle extract was recorded on binary and ternary mixtures of odd-numbered $C_{19:0}$ to $C_{29:0}$ synthetic *n*-alkanes (Table 2) of neighboring chain lengths. Except for *n*-$C_{19:0}$ and *n*-$C_{21:0}$, none of these alkanes were active when tested singly. *n*-$C_{21:0}$ was active at doses of 6 $\mu$g/cm$^2$ (50% reacting mites vs. 15% on the control; Fisher exact, $P < 0.05$) or higher (Figure 1). A mixture of the *n*-alkanes $C_{21}$, $C_{23}$, $C_{25}$, $C_{27}$, and $C_{29}$, at the proportions (0.2 : 3 : 7 : 12 : 5) close to that found in the TLC fraction F1A, was active at a total dose of 6 $\mu$g/cm$^2$, which corresponds to 4.7 leq *n*-alkanes (25% reacting mites vs. 7% on

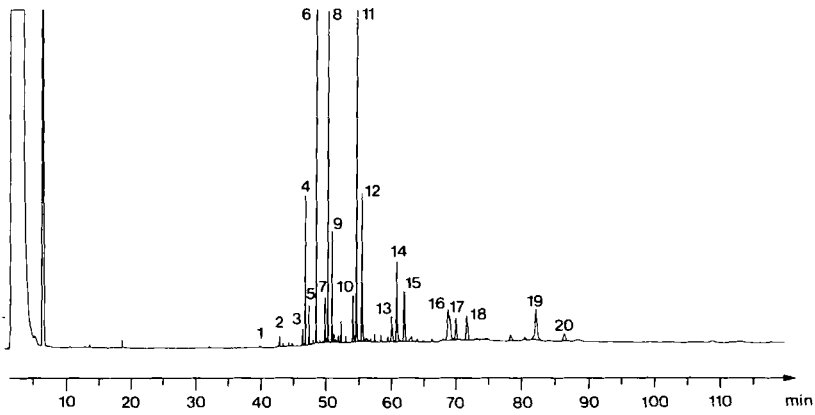


FIG. 2. Chromatogram (GC-FID) of cuticle extract of 8-day-old worker larvae on a nonpolar DB-5 high-resolution capillary column (see Methods and Materials). The numbered peaks (identified by GC-MS and retention time) show the following compounds: (1) $n$-$C_{19:0}$; (2) $n$-$C_{21:0}$; (3) $n$-$C_{23:1}$; (4) $n$-$C_{23:0}$; (5) $br$-$C_{23:0}$; (6) $n$-$C_{24:0}$ (internal standard); (7) $n$-$C_{25:1}$; (8) $n$-$C_{25:0}$; (9) $br$-$C_{25:0}$; (10) $n$-$C_{27:1}$; (11) $n$-$C_{27:0}$; (13) $n$-$C_{29:1}$; (14) $n$-$C_{29:0}$; (15) $br$-$C_{29:0}$; (16) $n$-$C_{31:1or2}$; (17) $n$-$C_{31:0}$; (18) $br$-$C_{31:0}$; (19) $n$-$C_{33:1or2}$; (20) $br$-$C_{33:0}$. Wax esters were only detected in GC-MS. In all, the $n$-alkanes (peaks 2, 4, 8, 11, 14, and 17) make up 48% of the products enumerated here. The extract was made up of 1.99 ($\pm$0.30) $\mu$g saturated and 0.69 ($\pm$0.12) $\mu$g unsaturated HCs. $n$-Alkanes were found to make up 1.27 ($\pm$0.24) $\mu g/cm^2$ and $br$-alkanes 0.73 ($\pm$0.09) $\mu g/cm^2$ of larva. $n$-$C_{19:0}$ was found below 1% and $n$-$C_{21:0}$, $n$-$C_{23:0}$, $n$-$C_{25:0}$, $n$-$C_{27:0}$, $n$-$C_{29:0}$, and $n$-$C_{31:0}$ were found in amounts of $\leq$ 0.03, 0.11 ($\pm$0.03), 0.35 ($\pm$0.15), 0.55 ($\pm$0.22), 0.21 ($\pm$0.09), and 0.09 ($\pm$0.6) $\mu g/cm^2$ of larva, respectively.

the control; $P < 0.05$, $N \geq 20$), or at higher amounts (27% and 41% reacting mites on 15 and 30 $\mu g/cm^2$, respectively). Addition of the attractant PA to the alkanes $n$-$C_{25:0}$ and $n$-$C_{27:0}$ either singly or to a mixture had no effect on the paraffins' activity, and PA alone was not active. This is consistent with the inactivity of TLC fraction F7 containing fatty acids. A mixture of three synthetic alkenes ($n$-$C_{19:1}$, $n$-$C_{21:1}$, and $n$-$C_{23:1}$), Vaseline, or a 12 leq dose of MP did not elicit a behavior response different from the solvent controls.

*Track Analysis.* The mean length per 0.2-sec vector (no-move vectors were excluded from calculations) increased with increasing doses of TLC fraction F1A on the treated area (Table 3), and a significant difference to the solvent control was observed at and above 1.2 leq (Tukey HSD, $P \leq 0.05$). The animals walked straighter on the treated area: the mean turn angle decreased significantly from 25° (log values reconverted into degrees) on controls to 19° on 5.9 leq. The analysis of parameters describing track segments from one border contact to the next confirmed the above results, i.e., mites increased walking speeds

TABLE 2. RESPONSE OF *Varroa* TO SYNTHETIC ODD-NUMBERED SATURATED AND UNSATURATED HYDROCARBONS ($C_{19}$–$C_{29}$) ON A CIRCULAR AREA[a]

| Compounds | Amount ($\mu$g) applied/cm$^2$ | Mites tested ($N$) | Runs ($N$) over at least one limit | Mites reacting (%)[b] |
|---|---|---|---|---|
| Single *n*-alkanes | | | | |
| Control | | 34 | 7 | 21a |
| $C_{19:0}$ | 30 | 23 | 13 | 57b |
| $C_{21:0}$ | 30 | 21 | 15 | 71bc |
| $C_{23:0}$ | 30 | 23 | 5 | 22a |
| $C_{25:0}$ | 30 | 30 | 10 | 33ab |
| $C_{27:0}$ | 30 | 50 | 11 | 22a |
| $C_{29:0}$ | 30 | 20 | 2 | 10a |
| Binary mixtures | | | | |
| Control | | 38 | 4 | 11a |
| $C_{19:0}$ and $C_{21:0}$ | 15 each | 22 | 13 | 59bc |
| $C_{21:0}$ and $C_{23:0}$ | 15 each | 21 | 16 | 76bc |
| $C_{23:0}$ and $C_{25:0}$ | 15 each | 21 | 17 | 81bc |
| $C_{25:0}$ and $C_{27:0}$ | 15 each | 41 | 30 | 76bc |
| $C_{27:0}$ and $C_{29:0}$ | 15 each | 21 | 7 | 33b |
| Ternary mixtures | | | | |
| Control | | 41 | 2 | 5a |
| Alkanes $C_{19:0}$–$C_{23:0}$ | 10 each | 45 | 30 | 67b |
| Alkenes $C_{19:1}$–$C_{23:1}$ | 10 each | 45 | 6 | 13a |

[a] For explanation, see Methods and Materials.
[b] Within test groups, percentages followed by different letters are significantly different at $P < 0.05$ (Fisher exact).

from 2.85 ± 1.1 mm/sec on controls to 4.06 ± 0.9 mm/sec on 5.9 leq of fraction F1A and decreased their angular velocity from 201.7 ± 82.3°/sec on controls to 179.2 ± 61.0°/sec on 5.9 leq.

The mites behaved essentially the same way on a substrate treated with *n*-$C_{21:0}$ as on one treated with fraction F1A. Higher vector lengths (Tukey HSD, $P < 0.05$) and speeds (not significant) as well as lower turn angles ($P < 0.05$) and angular velocities ($P < 0.05$) were recorded on $C_{21}$ compared to the solvent control (Table 3).

## DISCUSSION

*Varroa* shows an arrestment response on a substrate treated with a cuticle extract of 8-day-old worker honeybee larvae at a stimulus density corresponding to 12 leq. Of 98 arbitrarily chosen responses on the active cuticle extract, only two

TABLE 3. TRACK ANALYSIS OF PATHS MADE BY *Varroa* ON ARENA TREATED WITH NONPOLAR TLC FRACTION OF HONEYBEE WORKER LARVA CUTICLE EXTRACT (F1A) AND ONE OF ITS CONSTITUENTS, $n$-$C_{21:0}$[a]

| | | 0.2-sec vectors (mean ± SD) | | Path segments from one border contact to the next (mean ± SD) | |
|---|---|---|---|---|---|
| Stimulus | Amount applied/cm$^2$ | Vector length (mm)[b] | Log of turn angle[b,c] | Speed (mm/sec)[b] | Angular velocity (deg/sec)[b,d] |
| Control | | 0.53 ± 0.20a | 3.23 ± 0.87a | 2.85 ± 1.11a | 201.7 ± 82.3ab |
| F1A | 0.6 leq | 0.60 ± 0.28a | 3.25 ± 0.89ab | 3.15 ± 1.14ab | 231.1 ± 54.0ab |
| | 1.2 leq | 0.67 ± 0.32b | 3.16 ± 0.95ab | 3.66 ± 1.16b | 227.4 ± 62.3a |
| | 2.9 leq | 0.70 ± 0.31b | 3.01 ± 0.96b | 3.65 ± 1.03b | 193.8 ± 63.3ab |
| | 5.9 leq | 0.81 ± 0.33c | 2.95 ± 1.02b | 4.06 ± 0.90b | 179.2 ± 61.0b |
| Control | | 0.87 ± 0.38a | 3.10 ± 1.19a | 4.81 ± 1.3a | 272.5 ± 58.1a |
| $n$-$C_{21:0}$ | 30 μg | 1.05 ± 0.41b | 2.83 ± 1.25b | 5.58 ± 1.1a | 205.2 ± 68.2b |

[a]The differences, especially in the walking speeds, between the solvent controls for F1A and for $n$-$C_{21:0}$ show that a considerable variation exists between mites from different lots. For this reason behavioral activities of test solutions are only compared to the solvent controls made with mites of the same lot.

[b]Within groups, values followed by different letters are significantly different at $P < 0.05$ by Tukey HSD. This test permitted comparison between all treatments even though the number of returns was low for controls and 0.6 leq. The differences attributed serve above all to underline trends with increasing dose.

[c]Turn angles were calculated from running means generated over triads of consecutive 0.2-sec vectors.

[d]Angular velocities were calculated as the sum of the absolute turn angles made on the track between one border contact and the next one on the treated area.

responses were designated positive solely for having moved longer than 41.0 sec on the treated area. The 96 others were considered positive due to the fact that the number of returns at the borders of the treated area (either per 10 sec walk or per run) were above the 95% limits. Border recognition is therefore an outstanding feature of the response. The mite makes decisions at the border of the treated area that permit it to stay on the stimulus. This results in arrestment on the treated area. The most apolar TLC fraction F1 as well as the purified HC fraction F1A (devoid of wax esters) give similar results at equivalent stimulus doses. For the latter, a dilution series has been tested, with amounts of 0.6 leq (1.6 μg HCs) or higher being behaviorally active. HC fractions of the extract contain, for the most part, odd-numbered branched and straight-chain saturated and unsaturated HCs; removing the unsaturated compounds did not change the fraction's activity.

Synthetic compounds were only active when saturated HCs were present. Heneicosane at 30 μg/cm$^2$ elicited a response similar to the cuticle extract (Fig-

ure 1), but the behavioral threshold for this compound was at 6 $\mu g/cm^2$ or some 200 leq. Since TLC fraction F1A of cuticle extract showed activity at a level some 300 times lower (0.6 leq containing 0.02 $\mu g$ $n$-$C_{21:0}/cm^2$), the observed arrestment effect of the apolar fraction of cuticle extract is probably due a synergistic effect between the HCs it contains rather than to a single component. Heneicosane was active at much lower levels (0.04 $\mu g$) when presented in a mixture with odd-numbered $C_{23}$–$C_{29}$ alkanes. Since Vaseline was not active, it shows that the arrestment behavior and border recognition is more specific than just to the fatty texture of the substrate. Despite the fact that individual HCs differ in their physical properties on the membrane (as seen by their capacity to reflect light on the treated surface), *Varroa* appeared to be able to discriminate between them independent of this, i.e., some were active on their own whereas others were not (Table 2).

The lowest active dose of a mixture of synthetic $n$-alkanes imitating the proportions found in TLC fraction F1A was 6 $\mu g/cm^2$, but a dose of 0.6 leq of TLC fraction F1A containing 0.76 $\mu g$ $n$-alkanes was active. This suggests that the straight-chain alkanes do not alone account for the activity of the fraction, which also contains *br*-alkanes and alkenes. Both of the latter were inactive in terms of border recognition when tested alone, but notched box plot analysis showed that the *br*-alkanes significantly increased the duration of walking on the treated area compared to the solvent control due to the fact that *Varroa* often returned to the treated area after they had left it. Thus, apart from the presence of $n$-alkanes, the low behavioral threshold for fraction F1A depends, in addition, on the presence of *br*-alkanes. Monomethyl alkanes, which make up some 90% or more of the *br*-alkanes on adult bees, have been identified as mixtures of two or more 9-, 11-, 13- and 15-methyl alkanes (Francis et al., 1989), and our own GC-MS identification provided similar results for larval extracts. The straight-chain alkanes of our extracts could not be desorbed from the molecular sieve in sufficient purity (some branched material also migrated into the sieve) and were therefore not tested. TLC fraction F5 (fatty acid esters) prolonged the mites' stop times on the treated area (notched box plot analysis) suggesting that, in addition to the saturated HCs serving as cues for border recognition observed in our bioassay, further stimuli may control the mites' host recognition process.

Track analysis showed that *Varroa* walking on a homogeneously stimulating substrate move in a dose-dependent pattern. In general, the mites walk faster and straighter on higher doses of fraction F1A. Mites walking on 30 $\mu g/cm^2$ of heneicosane also walk faster and straighter than on the solvent control. Thus, synthetic $n$-$C_{21:0}$ elicits a similar response from the mites as the cuticle extract, a result that serves to confirm the importance of straight-chain odd-numbered alkanes as chemostimuli evoking the arrestment response in *Varroa*. These results indicate that chemostimuli from host cuticle not only influence the behavior of *Varroa* at the border of a treated area, but also affect *Varroa*'s walking behavior

on the treated substrate. Whereas 0.6 leq of fraction F1A was sufficient to evoke a response when considering criteria such as border recognition and the time spent walking on the treated area, 1.2 leq was necessary to evoke a significant response in walking speed and angular velocity.

Large day-to-day or lot-to-lot variability of mite behavior was observed throughout the study described here and was independent of the stimulus tested. A potential contamination of the membranes seems very unlikely considering the method of cleaning the Baudruche and treatment of the test area. Potential contamination by volatilization of some test compounds seems unlikely because day-to-day variations were observed even when the same batches of solutions were tested. The origin of the variations is most probably related to the mites' provenance.

Considering the extraction time of 15 min employed here, one may question the extent to which only compounds of purely cuticular origin were extracted. In other studies using the term "cuticular HCs", bees were extracted either for an unspecified time (McDaniel et al., 1984, Moritz et al., 1991, Nation et al., 1992) or for 10 min (Blomquist et al., 1980, Francis et al., 1989, Phelan et al., 1991). In this study, preliminary observations demonstrated that *Varroa* was arrested on a substrate rubbed with live larvae. In addition, the two major components of the extracts described here ($n$-$C_{25:0}$ and $n$-$C_{27:0}$) were also identified in volatiles from living larvae (Rickli et al., 1992), suggesting that the HCs originate from the exterior of worker larvae.

The tracks made by *Varroa* resemble those of other parasites and parasitoids, but with the difference that the mites increase speed and track straightness (doses $\geq$ 1.2 leq), while other arthropods usually decrease both in arrestment responses towards semiochemicals of their hosts (e.g., Waage, 1978) or conspecifics (Royalty et al., 1993). The walking pattern (fast and straight paths) and the net arrestment effect (arising from recognition of the border of the treated area and the return responses leading to highly increased periods of time spent on active substrates) seem to contradict each other. However, it might be explained by the behavioral context in which it operates. Two roles for contact chemoreception have been mentioned here for *Varroa*: for cell invasion and attachment to the bee larva during cocoon spinning. In both cases the success of the mite's response might depend on the speed of the reaction. The speed with which *Varroa* responds during cell invasion might contribute to avoiding detection by the host, which would lead to removal of the parasite from the colony, as in the case of the original Asian host, *Apis cerana* (Peng et al., 1987; Büchler et al., 1992). The function of speed to avoid being crushed between the larval body and cell wall during cocoon spinning by the bee is obvious.

Alkanes are widespread as chemostimuli in arthropods. In *Acarus immobilis* (Acarina) $C_{13}$, $C_{25}$, $C_{27}$, and $C_{29}$ HCs are employed to attract females to the vicinity of males (Sato et al., 1993). It is significant that *Acarpis woodii*, a mite

which invades the tracheae of adult honeybees, shows an arrestment response on cuticular HCs of its host and, similar to *Varroa*, especially to alkanes (Phelan et al., 1991). Honeybees themselves can be trained to discriminate between $C_{23:0}$ and $C_{25:0}$ and even between their mixtures (Getz and Smith, 1987). Further, application of hexa- and octadecane increases aggressive behavior between hivemates (Breed and Stiller, 1992). Therefore, alkanes seem to be implicated in the nestmate-recognition process of bees, and the same is proposed for other social insects such as wasps, ants, and termites (Singer and Espelie, 1992, and references therein). Bumblebees, *Bombus terrestris*, mark visited flowers with a secretion from the tarsal glands containing $C_{19}$–$C_{31}$ HCs; only when both saturated and unsaturated compounds were combined could a response similar to natural scent marks be observed, but alkanes alone could induce a part of the response (Schmitt et al., 1991). In the parasitoid *Trichogramma brassicae*, a blend of odd-numbered $C_{21:0}$–$C_{29:0}$ from host egg masses stimulates oviposition (Grenier et al., 1993).

Saturated and unsaturated HCs present on honeybees are also present on *Varroa* (Nation et al., 1992; our own unpublished results), a factor which may serve to reduce detection of the parasite via mimicry. The presence of these compounds to which *Varroa* is sensitive on the parasite's own cuticle may also contribute to the mutualistic relationship between members of the same and different families in single and multiinfested brood cells (Donzé and Guerin, 1994). *Varroa* and honeybees may, in fact, use the same compounds as semiochemicals, as demonstrated by the fact that fatty acid esters that attract *Varroa* to host larvae of a particular age are also employed to trigger cell capping in worker bees (LeConte et al., 1990). The same could be true for saturated HCs, i.e., alkanes are suggested to function as semiochemicals in bees for nestmate and age-recognition (Getz et al., 1989) and are simultaneously employed for host recognition by *Varroa*.

Straight-chain alkanes are ubiquitous within the hive, on adult bees (Francis et al., 1989; our own unpublished results) as well as on beeswax (Tulloch, 1980; our own unpublished results). Indeed, extracts of beeswax and adult bees cause arrestment of *Varroa* on the semipermeable membrane employed here (unpublished results). We must therefore conclude that *Varroa* can recognize bee larvae using HCs only if the relative proportions of saturated HCs employed are sufficiently specific to this lifestage, or in combination with other cues (chemical or otherwise) peculiar to the lifestage it parasitizes within brood cells. It is noteworthy that *br*-alkanes, which caused *Varroa* to walk for longer durations in our bioassay, make up 27% of the hexane-soluble material on larvae but constitute less than 7% of similar extracts from cell walls or from adult bees of two days or older (Francis et al., 1989, our own unpublished results).

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