

Aliphatic Alcohols and Aldehydes of the Honey Bee Cocoon Induce Arrestment Behavior in *Varroa jacobsoni* (Acari: Mesostigmata), an Ectoparasite of *Apis mellifera*

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The ectoparasitic mite *Varroa jacobsoni* reproduces in the capped brood of the honey bees *Apis cerana* and *Apis mellifera*. Observations on the reproductive behavior of the mite have shown a well-structured spatial allocation of its activity using the bee or cell wall for different behaviors. The resulting advantages for the parasite of this subdivision of the concealed brood environment suggests an important role for chemostimuli in these substrates.

Extracts of the European honey bee cocoons induce a strong arrestment response in the mite, as indicated by prolonged periods of walking on the extracts applied on a semipermeable membrane and by systematically returning to the stimulus after encountering the treatment borders. Two thin-layer chromatography fractions of the cocoon extract eliciting arrestment were found to contain saturated C₁₇ to C₂₂ primary aliphatic alcohols and C₁₉ to C₂₂ aldehydes.

We analyzed extracts of the cocoon and different larvae, pupae, and adults of both worker and drone *A. mellifera* to determine the relative amounts of these chemostimuli in the different substrates employed by *Varroa*. Both aldehydes and alcohols were more abundant in the cocoon than in the cuticle of adult or developing bees.

Mixtures of the aliphatic alcohols and aldehydes at the proportions found in the cocoons acted synergistically on the arrestment response, but this activity disappeared when mixed in equal amounts. When these oxygenated chemostimuli were mixed with C₁₉ to C₂₅ alkanes at the proportions found in the cocoon extract, we observed a significantly lower threshold for the chemostimulant mixture. These results indicate how *Varroa* may use mixtures of rarer products to differentiate between substrates and host stages during its developmental

Abbreviations used: C_{18:0} = octadecane; C₁₈-OH = octadecanol; C₁₈-CHO = octadecanal; C₁₈-COOH = octadecanoic acid; cceq = comb cocoon equivalent; CH₂Cl₂ = dichloromethane; F₁-F₆ = TLC fractions of cocoon extract; FID = flame ionization detector; gceq = glass cocoon equivalent; GLC = gas-liquid chromatography; MS = mass spectroscopy; TLC = thin-layer chromatography.

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cycle within honey bee brood cells. *Arch. Insect Biochem. Physiol.* 37:129–145, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

The ectoparasitic mite *Varroa jacobsoni* originally parasitized the Asian honey bee, *Apis cerana*, and is currently causing severe damage worldwide to the European honey bee, *Apis mellifera*. The adult female is the only stage which parasitizes adult honey bees, preferring nurse bees (Hoppe and Ritter, 1988; Kraus, 1990, 1993; Steiner, 1993). In order to reproduce, *Varroa* females infest the brood by walking first along the cell rim, then the cell wall, and finally by crawling between the larva and the cell wall into the larval food at the base of the cell. *Varroa* shows a high preference for drone larvae (Tewarson et al., 1992; Fuchs, 1990) and invades brood cells only in the hours just before operculation, a process which suggests recognition of host larval sex and age by the female mites. Indeed, Le Conte et al. (1989) identified three fatty acid esters (methyl palmitate, ethyl palmitate, and methyl linolenate) as attractive to *Varroa*. The quantity of esters available on drone larvae is much higher than that on worker larvae and increases rapidly before capping of the cell (Trouiller et al., 1992; Le Conte et al., 1994). In addition, palmitic acid, present in volatile extracts of worker larvae, elicited upwind walking in *Varroa* on a servosphere (Rickli et al., 1992). Apart from the volatiles cited above, saturated straight-chain odd-numbered alkanes (C₁₉–C₂₉) synergise to mediate arrestment of mites (Rickli et al., 1994).

In the capped brood, *Varroa* mites show specialized behaviors, using different sites in the cell to perform different activities. After operculation, the bee larva spins its cocoon on the cell wall. At the beginning of this period, *Varroa* females are always observed on the mobile bee larva but the proportion of time the infesting female, and, later, the offspring, spend on the cell wall increases. When hungry, the mites move to the bee larva and feed on a single feeding site prepared by the mother. Soon after each feeding bout they return to the fecal accumulation on the posterior part of the cell wall (Donzé and Guerin, 1994; Donzé, 1995). This concentration of mite activity allows the infesting fe-

male to lay her eggs, and the developing mites to molt, on the undisturbed parts of the cell wall (Donzé and Guerin, 1997).

The complex behavior observed in the capped brood suggests that chemostimuli, among other cues, are involved in the recognition of the several sites used. In the present study, we analyzed the reaction of female mites to extracts of the honey bee cocoon and identified chemostimuli in these extracts. Furthermore, to better understand the use of chemostimuli during cell infestation and in space structuring within the capped brood by *Varroa*, we quantified the amounts of the chemostimuli in the cocoon and on the cuticle of the honey bee at different stages of its life cycle.

MATERIALS AND METHODS

Mites

Lots of sixty to eighty infected adult bees were collected from heavily infested colonies, held for one to six days in the dark at room temperature, and fed with candy. For each test, one *Varroa* mite was removed from a bee and tested within a few minutes.

Extracts of Biological Origin

Cocoons From Brood Combs. Cocoons were obtained from noninfested young worker prepupae in old combs in which a high amount of brood had developed. One comb cocoon equivalent (ccec) represents the unknown number of cocoons, resulting from the successive occupancy of the cell, extracted from one cell (without cell base). The cocoons were then immersed for 30–120 min at room temperature in dichloromethane (all reagents were of analytical grade) using 1 ml per 10–20 cells. The resulting extract was filtered through glass wool in a Pasteur pipette.

Cocoons Spun on Glass. In order to have a defined number of cocoons in the extract and to exclude possible contamination from the wax underlying the cocoons, we extracted cocoons spun on glass. Worker or drone combs with larvae ready for operculation were placed above a

clean glass plate in an incubator at 34°C, 60% relative humidity. When the larvae began to spin their cocoons in the unsealed cells they fell out onto the glass plate. Fifty larvae were transferred to a crystallizing dish ($\varnothing = 20$ cm) where they spun their cocoons on the glass walls. The dichloromethane extracts of these cocoons also contained compounds of larval excrement. One glass cocoon equivalent (gceq) represents the cocoon spun by one larva.

Bee Cuticle Extracts. One hundred noninfested drone or worker developmental stages (i.e., larvae at the time of cell capping, prepupae, pupae with white eyes (6 and 5 days post-capping for drone and worker pupae) and pupae with yellow-brown bodies (12 and 9 days post-capping for drone and worker, respectively)) were extracted twice in 8–10 ml dichloromethane for 20 min. In addition, 100 young adult drones and workers which had just emerged in an incubator, as well as older adult drones and workers sampled at the beehive entrance, were extracted twice in 8–10 ml solvent for 20 min.

Fractionation and Analysis of Extracts

Thin Layer Chromatography (TLC). Details of the TLC procedures are described in Rickli et al. (1994). We loaded 10–12 cceq on 3/4 of the TLC plate width and a series of synthetic standards on the side (Fig. 1). The visualized fractions were scraped from the plate, extracted in dichloromethane and concentrated to 500 μ l. The fractions F₁–F₅ (including the nonmigrating material) were tested for biological activity in the bioassay and their constituents analyzed by GLC-MS. A strip (F₆) situated under the application band was used as a control to test for the biological activity of the silica gel and solvent.

Column Chromatography. As recoveries of the synthetic standards from TLC plates proved low (about 30%), biologically active extracts were column chromatographed on silica gel (70–230 mesh) in a Supelco glass column (id. 10 mm, Nr. 6-4756) to quantify active products of the different honey bee instars and of the cocoons spun on glass. The silica gel was successively washed with chloroform/methanol (2:1), diethylether and hexane, and then heated for 12 h at 120°C. One ml of concentrated extract corresponding to 20 individuals or gceq was deposited on a 5 g column of silica gel. Elution of cocoon constituents was made with the following mixtures of diethyl ether in hexane (v/v%): 0% (15 ml), 5% (20 ml), 7% (10 ml), 8% (20 ml),

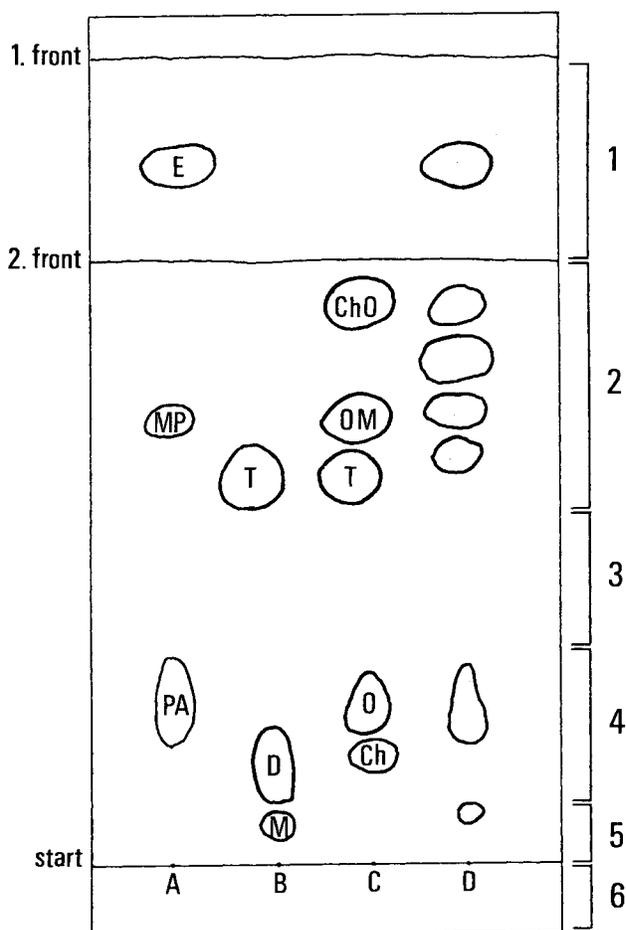


Fig. 1. Fractionation scheme by thin-layer chromatography of a honey bee cocoon extract (D), and the following synthetic standards (20–100 μ g). A: Mixture of palmitic acid (PA), methyl palmitate (MP), and eicosane (E). B: Mono- (M), di- (D), and tri-glycerid oleates (T). C: Cholesterol oleate (ChO), methyloleate (OM), glycerintriolate (T), oleic acid (O), and cholesterol (Ch). D: Zones 1–6 indicate the fractions F₁–F₆ of the cocoon extract tested for their biological activity. F₁ is the most apolar fraction and F₅ the nonmigrating material on, or close to, the application band. F₆ was situated under the application band and was used as control for silica gel and solvent activity. 1. Front: solvent front after the first two runs, i.e., hexane and toluene. 2. Front: solvent front after the third run, i.e., a 70:3:1 mixture of hexane/diethylether/acetic acid. Silica gel TLC plates were 20 × 20 cm with 0.2 mm layer thickness (Merck 5748).

15% (10 ml), 50% (10 ml), 100% (20 ml). Since the composition of bee extracts was less complex, it was eluted with the following mixtures of diethyl ether in hexane: 0% (15 ml), 8% (10 ml), 100% (20 ml). Fractions of about 5 ml each were collected, dried under vacuum, redissolved in 500 μ l toluene, and held in the freezer until use. The mean recoveries of the different functional groups were calculated with known

amounts of synthetic standards subjected to column chromatography as above, and were $93 \pm 3\%$ for alkanes, $96 \pm 8\%$ for alcohols and $99 \pm 7\%$ for aldehydes ($n = 3$, mean \pm SD).

Identification and Quantification. A Hewlett Packard HP5890 gas chromatograph equipped with an HP5971A mass selective detector (GLC-MS) was employed to identify constituents of biologically active TLC fractions and to confirm the structures of the substances in the quantified column chromatographed fractions. 1–1.5 μ l samples were injected on-column (uncoated precolumn) onto either a 30 m XTI5-5 (Restek 0.25 mm ID, 0.25 μ m film) or a 15 m DB5.HT (J+W 0.25 mm ID, 0.15 μ m film) fused silica capillary columns. The columns were temperature programmed from 60°C at 10°C/min to 350°C, 350°C for 10 min with He as carrier gas. The mass selective detector, run in the EI mode at 190°C, was set to a scan range of m/z 50–650. Mass spectra and retention times were compared with those of synthetic standards.

Gas chromatography with flame ionization detection (GLC-FID) was used to quantify the bioactive compounds in the cocoons and on the cuticle of honey bee larvae, pupae, and adults. 1 μ l samples were injected splitless on two columns of different polarity: 30 m DB5MS 0.32 i.d. with 1 μ m film (non-polar) and 30 m DB35MS 0.25 i.d. with 0.25 μ m film (mid-polar) with H₂ as carrier gas. The following temperature programs were employed: for alcohols—3 min at 80°C and then 10°C/min to 300°C; for esters and aldehydes—3 min at 80°C and then 10°C/min to 200°C, 1°C/min to 220°C, 2°C/min to 250°C. The quantification of a substance was considered as valid only when the results from both columns corresponded. A single extract of each honey bee instar and cocoon types was quantified.

Synthesis of Aldehydes

Aliphatic aldehydes from the corresponding primary aliphatic alcohols (Sigma, Buchs, Switzerland; purity >98%) were synthesized using the method of Santaniello et al. (1978), which consists of oxidation of alcohols by chromic acid adsorbed on silica gel (SiO/H₂CrO₄). The purity of the synthesized aldehydes analyzed by GLC on the DB5 column was >95% and the quantity of corresponding alcohols <0.3%.

Bioassay

Bioassay Arena. The bioassay used here was adapted from Rickli et al. (1994). A transparent

semipermeable biological membrane (baudruche; John Long, Inc., Belleville, NJ, USA) washed in hexane and acetone was stretched over a small water bath at 32.5°C, providing a humidity of 85–95% at the membrane surface. A white plastic plate equipped on the upper side with sagex floats and with three concentric circles of 12, 24, and 36 mm in diameter was placed under the membrane. The floats were thick enough to avoid contact between the membrane and the plastic plate and both the center and borders of the plastic plate were pierced with 5 mm holes to ensure circulation of water. The test solutions were applied on the semipermeable membrane between the inner and middle circles. The treated area (3.4 cm²) was about 1.7 times larger than the surface area of the worker cell wall (excluding the hemispheric cell base). Each test solution was assayed with mites of at least two different lots on different days. A single mite was deposited with a fine brush in the center of the arena and the test was terminated when the mite left the outer circle or after 3 min, whichever came first (Fig. 2).

Testing Behavioral Responses of *Varroa* to Synthetics. Synthetic compounds tested were dissolved in either pure dichloromethane or in a mixture of dichloromethane containing 20–30% hexane if the compound had a carbon chain greater than C₂₂. Following GLC-MS analysis of cocoon extracts, series of synthetic analogs were tested according to their functional groups. If the total mixture induced a behavioral response, the products were tested in binary or quaternary mixtures and then tested singly. This permitted recognition of possible synergism between compounds within a chemical group. Details of the doses are given in the tables and figures. Dose-response curves were established with the active compounds, which allowed us to determine the threshold level at which the mites' response appears on the membrane assay.

In addition to compounds reported in the tables and figures, a mixture of 2-pentadecanone, 2-heptadecanone, and 2-nonadecanone (Fluka, >97%) as well as mixtures of fatty acid methyl- and ethyl-esters (C₁₆ to C₂₀) and a mixture of five saturated fatty acids (C₁₈–C₂₂-COOH) were also tested at 1.2, 6, and 18 μ g/cm².

Possible synergism between active alkanes, alcohols, and aldehydes was tested by taking a pair of products from each functional group. Behavioral responses to solutions containing different proportions of alcohols and aldehydes were

tested. For this a solution containing an equal proportion (1:1) of the two alcohols, octadecanol and eicosanol, and the two aldehydes eicosanal and docosanal, was used. The response to this mixture was compared with a second solution (10:1) containing ten parts of the two alcohols and only one part of the two aldehydes. The latter solution was thus tested at lower doses (Table 3).

In an effort to reconstitute a cocoon extract containing almost all contact chemostimulants identified, we used approximate quantities contained in the cocoons spun on glass (Table 3). Heptacosane was purposely omitted because of its high amount in the cocoons, in an effort to avoid masking the minor constituents. The aldehydes were slightly overestimated. In order to estimate the behavioral importance of hydrocarbons, which represented a high proportion of the complete cocktail, a second mixture of synthetics was made up containing only the C_{19:0} to C_{25:0} *n*-alkane series. These synthetic solutions were compared with extracts of worker cocoons spun on glass and with extracts of worker cocoons sampled in combs.

Data Analysis. Assays conducted on series of fractions or synthetics accompanied by a solvent control and the brood comb cocoon extract (positive control) on the same day were subjected to batchwise statistical analysis. In view of the day-to-day variation in the mites' responses to active extracts (Rickli et al., 1994), each test series on a day began with a test of the positive control on a group of mites (0.75–1.7 comb cceq). When less than 50% of mites showed arrestment to the extract, no other tests were made with that batch of mites.

Test runs were recorded on VHS video from a camera held perpendicularly above the membrane and behaviors of the mites were simultaneously recorded using The Observer (Noldus Information Technology, The Netherlands). The time a mite walked and stopped on the different zones of the arena were quantified. The number of contacts made by mites on the treated membrane with the borders of the treated zone, and the number of subsequent returns toward the treated zone, were counted. Records from 440 such runs on the solvent control were pooled and used to define a standard for the parameters walking time in the treated zone and returns at its border. In all, 95% of the mites tested on the solvent control walked less than 50 s on the treated area and made fewer than four returns per run. A mite was therefore considered as reacting to the test when

it showed a response higher than the 95% limits in one of the parameters (i.e., when it walked longer than 50 s on the treated area or when it proceeded to make at least four returns at the treatment borders). The proportion of reacting mites to a given solution was compared with Fisher's exact test to the proportion of reacting mites on the solvent control and with the cocoon extract tested on the same day. In addition, the intensity of the arrestment response of the reacting mites to each treatment was scaled using the mean number of returns at the borders of the treated zone and compared with one another after transformation (natural logarithm). These data were analyzed by ANOVA and Tukey's multiple comparison test. The intensity of the behavioral response was compared only between treatments which induced a significantly higher proportion of mites to respond than on the control solution. This avoided inclusion in the analysis test groups showing small numbers of reacting mites.

RESULTS

Varroa's Responses to Cocoon Extracts

Varroa females showed a strong arrestment response to a dichloromethane extract of bee cocoons (Fig. 2). Depending on the lot of mites tested, 20–100% of *Varroa* showed responses over one of the 95% limits set for the solvent control mites. No reason could be found for this variation. The most apolar TLC fraction F₁ (Fig. 1) did not show any significant activity; only 13% of mites reacted to this fraction even though it contains the alkanes present on the bee larval cuticle which arrest *Varroa* (Rickli et al., 1994). The two fractions F₂ and F₄, respectively, induced 52% and 58% of mites to respond. At the 4.5 cceq dose, the proportion of reacting mites did not differ significantly from the cocoon extract (Table 1). Fractions F₃ and F₅ elicited responses no different from the control runs. When the four bands of fraction F₂ were scraped separately off the TLC plate, a weak arrestment response to the three most polar bands was sometimes recognizable, but the responses were neither very clear nor very repeatable to draw conclusions. This did indicate, however, that the active substances were probably spread over several bands of F₂. A reconstituted cocoon extract comprising a solution of all TLC fractions of the cocoon extract was as active as the crude extract, when tested at three times the dose of the cocoon extract.

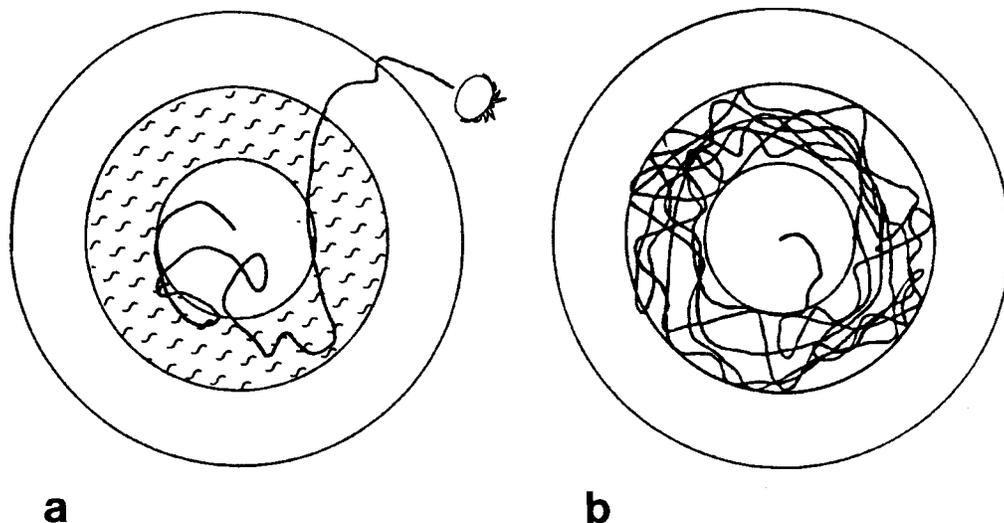


Fig. 2. *Varroa* tracks on a semipermeable membrane with an extract of a honey bee cocoon applied between the inner and middle circles. The mite was released in the center and observed until it left the outer circle or after 3 min, whichever came first. (a) On the control, the mite walked for 19 s on the solvent

treated area and did three returns after encountering the treated zone borders between the inner and middle circles. (b) On a crude extract of 0.5 cceq/cm^2 , the mite walked for 134 s and made 33 returns to the zone treated with the cocoon extract after encountering the borders.

Saturated straight-chain alkanes, fatty acid esters, and palmitic acid co-migrated with the cocoon TLC fractions F_1 , F_2 , and F_4 , respectively. Arrestment activity observed for cocoon fractions could have been due to compounds already known to affect *Varroa* behavior, eicosane, methyl palmitate, ethyl palmitate, methyl linolenate, and palmitic acid were first compared with the total cocoon extract and with fractions F_2 and F_4 (Table 1). Eicosane elicited an arrestment response in 72% of mites, but the fatty acid esters and palmitic acid elicited only very weak responses.

After separation of the cocoon extract by column chromatography, a clear arrestment activity by the mites was observed in the fraction which contained the hydrocarbons. Apart from this hydrocarbon fraction, there was quite an amount of correspondence between the response to the TLC and the column chromatography fractions in terms of the chemical classes causing *Varroa* arrestment.

Chemical Analysis of Cocoon Extracts

The TLC fraction F_1 , which migrated the fastest, contained *n*-alkanes, branched alkanes, and alkenes from C_{18} to C_{33} as well as a large amount of long-chain wax esters. Fraction F_2 contained $C_{16:0}$, $C_{16:3}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{20:1}$, $C_{20:2}$, $C_{20:3}$, $C_{22:0}$, and $C_{24:0}$ fatty acid methyl esters and $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ fatty acid ethyl esters. F_2 also contained long-chain

wax esters composed of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{20:0}$ fatty acids esterified to even-numbered C_{16} to C_{32} alcohols, along with a small quantity of saturated even-numbered C_{16} to C_{30} aliphatic aldehydes. Odd-numbered aldehydes along with unidentified branched and unsaturated aldehydes were also present at amounts too low to allow definite identification. F_2 also contained 2-heptadecanone and 2-nonadecanone and a host of other unidentified compounds.

The more polar active TLC fraction F_4 contained even-numbered C_{14} to C_{30} aliphatic alcohols, and the presence of odd-numbered ones at lower quantities was indicated by GLC-MS. After esterification, fraction F_4 was also shown to contain the fatty acids $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{19:0}$, $C_{20:0}$, $C_{24:0}$, $C_{25:0}$, $C_{26:0}$, $C_{27:0}$, $C_{28:0}$, $C_{30:0}$, $C_{32:0}$, and $C_{34:0}$ by GLC-MS. Finally, F_4 also contained a series of unidentified compounds of molecular weight >600 , possibly hydroxylated wax esters (e.g., diols or hydroxy acids). Since fractions F_3 , F_5 , and F_6 elicited behavioral responses no different from the control, their constituents were not analyzed.

Quantification of Behaviorally Active Compounds

Compounds which elicited behavioral responses on the baudruche membrane were quantified following fractionation by column chromatography (Fig. 3). Saturated *n*-alkanes

TABLE 1. Responses of *Varroa* on a Baudruche Membrane to an Extract of Honey Bee Cocoon, to Fractions of this Extract Obtained by Thin-layer Chromatography and to Eicosane, Palmitic Acid, and Fatty Acid Ester Constituents of the Extract

Test material	Quantity on treated area ^b	N <i>Varroa</i> tested	% reacting <i>Varroa</i> ^c	Fisher's exact test compared ^d to:		Intensity of reaction	
				cocoon	control	Returns ^e mean ± SEM	Tukey's test
^a Control = fraction F ₆		25	16	****		5.3 ± 2.4	/
Cocoon from comb ^b	2.2 cceq	21	81		****	20.6 ± 3.7	a
^a Fraction F ₁	4.5 cceq	24	13	****	n.s.	9.7 ± 7.1	/
^a Fraction F ₂	4.5 cceq	21	52	n.s.	*	13.1 ± 1.8	ab
^a Fraction F ₃	4.5 cceq	24	0	****	n.s.	0	/
^a Fraction F ₄	4.5 cceq	26	58	n.s.	*	8.4 ± 1.7	b
^a Fraction F ₅	4.5 cceq	25	12	****	n.s.	3.7 ± 1.2	/
Control CH ₂ Cl ₂	50 µl	24	0	****		0	/
Cocoon from comb ^b	1.7 cceq	18	83		****	32.9 ± 5.7	a
Eicosane (C _{21:0})	60 µg	25	72	n.s.	****	14 ± 2.3	b
Control CH ₂ Cl ₂	50 µl	21	0	****		0	/
Cocoon from comb ^b	1 cceq	19	84		****	35.6 ± 4	a
Esters ^f	20 leq	24	0	****	n.s.	0	/
Esters ^f	200 leq	24	25	***	*	4.6 ± 0.4	b
Palmitic acid	10 µg	10	10	***	n.s.	10	/
Palmitic acid	100 µg	22	14	****	n.s.	3.3 ± 1.8	/
^a Fraction F ₂	3 cceq	24	54	n.s.	****	14.8 ± 3.6	b
^a Fraction F ₄	3 cceq	24	46	*	***	7.8 ± 0.8	b

^aCocoon TLC fractions F₁–F₆ as defined in Figure 1.

^bThe treated area of the membrane measured 339 mm². One cceq represents the extract of the cocoon from one cell in a comb.

^cA *Varroa* was considered as “reacting” to the test material if it walked for ≥50 s on the treated area or if it made at least four returns toward the treated area on encountering the border.

^dFisher's exact test: n.s. = non-significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

^eMean number of returns per run. Only reacting mites were considered when calculating the means ± SEM for the ANOVA and Tukey multiple comparison test. The numbers of returns were transformed (natural logarithm) prior to statistical analysis. For each experimental series, the treatments assigned a different letter differ significantly at *P* < 0.05.

^fOne leq indicates the quantity of esters extracted from the 8-day-old larval cuticle (after Le Conte et al., 1989), i.e., 0.26 µg ethyl palmitate ; 0.09 µg ethyl palmitate and 0.59 µg methyl linolenate.

constituted the major chemical group in all extracts. Some 42 and 58 µg *n*-alkanes were recovered from each worker and drone cocoon spun on glass. The quantity on a single worker and drone larva prior to cell capping was 2.8 and 1.9 µg. The quantity of *n*-alkanes on the cuticle increases with the age of the developing bee. For most developmental stages, as well as for the cocoon, the C₂₅–C₂₉ alkanes were present in the highest proportions, with C₂₇ and C₂₉ constituting 40–80% of *n*-alkanes extracted (Fig. 3a). Heneicosane, which induces an arrestment response in *Varroa*, was represented at a higher proportion on the pupae and young adult cuticle of both sexes than on the precapped larvae, the prepupae, or the cocoons.

Drone and worker cocoon extracts contained 4.5 and 3.8 µg aliphatic alcohols, respectively. Long-chain alcohols (>C₂₂) occur in higher quantities. C₁₈ and C₂₀-OH, which evoke a strong

behavioral response from *Varroa*, represent about 13 and 8% of the quantified alcohols, respectively (Fig. 3b). Compared to the cocoon extracts, all alcohols, and particularly the shorter ones, were present in small amounts in almost all extracts of bees. The only behaviorally active alcohol which was regularly found on the different bee instars was octadecanol.

Drone and worker cocoon extracts contained 72 and 81 ng C₁₈–C₂₂ aliphatic aldehydes, respectively, and octadecanal represented about 80% of these. C₂₄–C₃₀ aldehydes were not quantified but preliminary results indicated C₂₆ and C₂₈ aldehydes to be the more abundant ones in the cocoon. The C₁₈ and C₂₀ aldehydes were also present on the prepupal and pupal cuticle but were absent from the larval and adult cuticle. As the aldehydes are not very stable, these values could be an underestimation.

Each bee instar and cocoon can be charac-

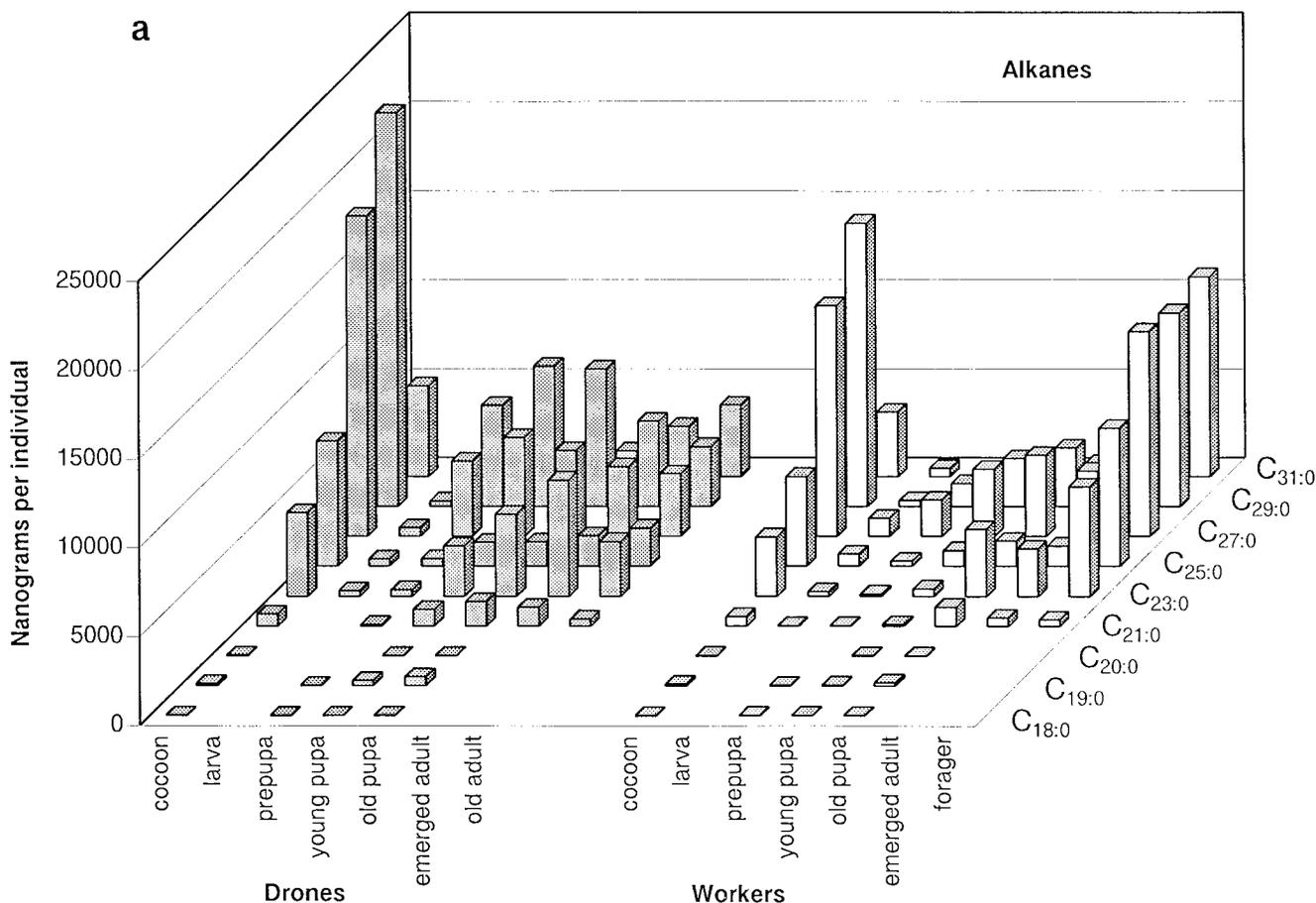


Fig. 3. Quantities of primary alkanes, aldehydes, and alcohols in extracts of honey bee worker (white histograms) and drone (shaded histograms) instars, as well as their cocoons spun on glass. (a) Saturated alkanes; (b) primary aliphatic aldehydes (e.g. 20-CHO for eicosanal) and primary aliphatic

alcohols (e.g., 20-OH for eicosanol). The values indicated are ng per individual or per cocoon. The detection limit was 1 ng and zero values are omitted. See Materials and Methods for further information.

terized by its pattern of quantified lipids. For this we used a multivariate analysis of the principal components which showed that both worker and drone cocoons were clearly distinguished from all bee instars. The first principal component accounted for 51% of the total variance and allowed discrimination between the cocoon extracts (weighted 1.3 for worker and 2.0 for drone) from all bee cuticle extracts (weighted from -0.65 to 0.1). The second principal component absorbed 16% of the total variance. Identical results were obtained when we included only the compounds which induced arrestment of *Varroa* females on the baudruche membrane.

Responses of *Varroa* to Synthetics

Alcohols. The behavioral responses of female *Varroa* were first bioassayed on mixtures of the eight even-numbered aliphatic alcohols,

i.e., C_{16} to C_{30} -OH (results not presented). Tests with increasing doses (i.e., 1.2, 6, 18, and $72 \mu\text{g}/\text{cm}^2$ each) showed an optimal response at $18 \mu\text{g}/\text{cm}^2$ each, with 68% of reacting mites ($n = 28$) walking 94 ± 13 s on the treated area, versus 4% on the control, as against 90% of reacting mites walking 107 ± 11.3 s on the crude cocoon extract. To identify which particular alcohols induced the behavioral response, we tested them in binary mixtures (Table 2) and singly (Fig. 4). Both even and odd aliphatic alcohols from heptadecanol to docosanol induced an arrestment response in 46–80% of female *Varroa* at an average response intensity of 10.7 ± 2 to 25.5 ± 3.7 returns per run ($n > 21$). Octa- and nonadecanol induced the best response. No clear activity was observed with mixtures of C_{24} to C_{30} -OH. To detect *Varroa*'s behavioral threshold, a quaternary mixture of hexadecanol, octa-

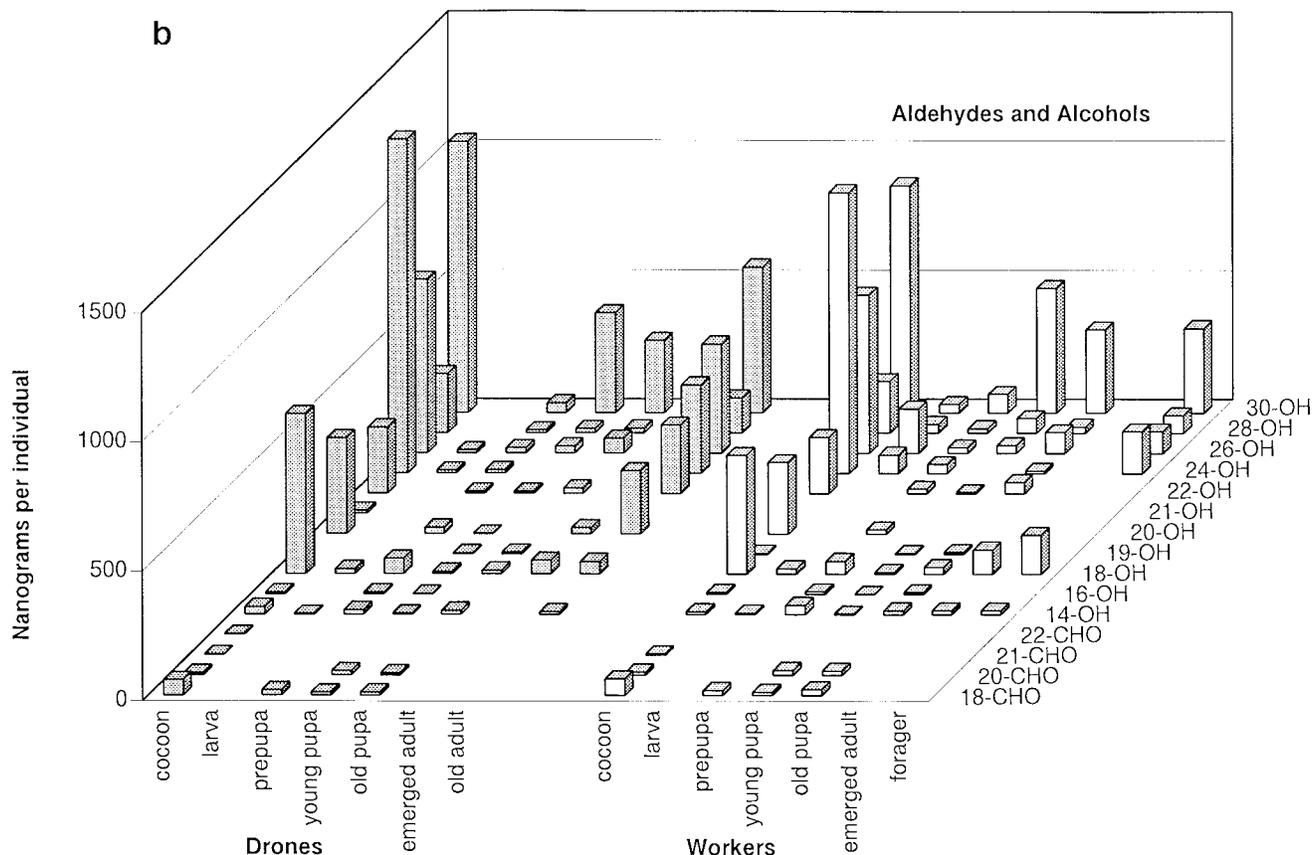


Figure 3b.

decanol, eicosanol, and docosanol was tested at low doses (Fig. 6). Increasing the dose from $0.6 \mu\text{g}/\text{cm}^2$ each to $1.8 \mu\text{g}/\text{cm}^2$ each resulted in an increase in the proportion of reacting mites from 29% ($n = 21$) to 47% ($n = 30$), while a further increase in dose to $6 \mu\text{g}/\text{cm}^2$ each resulted in a clearly stronger arrestment with 56% ($n = 25$) of mites showing 17.5 ± 3.2 returns at the border of the treated area.

Aldehydes. The mixture of eight even-numbered aliphatic aldehydes C_{16} to $\text{C}_{30}\text{-CHO}$ induced an optimal response at $3 \mu\text{g}/\text{cm}^2$ each with 70% of reacting mites ($n = 20$) walking 123 ± 13.8 s on the treated area (mean \pm SEM), versus 8.3% on the control, and 100% on the crude cocoon extract (126 ± 20 s of displacement). The four-component mixture of hexadecanal, octadecanal, eicosanal, and docosanal at $5.3 \mu\text{g}/\text{cm}^2$ each on the membrane induced strong arrestment in 75% of mites, while its activity dropped to 33% when tested at $3 \mu\text{g}/\text{cm}^2$ each (Table 2). By comparison, the quaternary mixture of tetra-, hexa-, octacosanal, and tricon-

tanol induced only a weak behavioral response. However, this weak response seemed to indicate that these longer aldehydes had some role in the 8 aldehyde mixture. In spite of these considerations, only the hexadecanal to tetra-

cocanal products were tested singly in the membrane bioassay (Fig. 5). In this series, all even and odd aldehydes from nonadecanal to docosanal induced strong arrestment in 52–80% of the mites. The behavioral response induced by eicosanal was particularly remarkable, as 80% of mites walked for 131 ± 12 s on the treated area during the 180 s of bioassay ($n = 25$). The behavioral threshold was tested with a binary mixture of eicosanal and docosanal at 0.6, 1.8, and $6 \mu\text{g}$ each/ cm^2 (Fig. 6). It induced behavioral responses in 23, 58, and 71% ($n = 22$) of *Varroa*, respectively. The mean arrestment intensity increased from 5.2 ± 1 to 17.8 ± 3.1 returns when the aldehyde dose applied passed from $0.6 \mu\text{g}/\text{cm}^2$ each to $1.8 \mu\text{g}/\text{cm}^2$ each.

It is clear from these tests that more than $7.2 \mu\text{g}/\text{cm}^2$ of the alcohol mixture and more than $1.2 \mu\text{g}/\text{cm}^2$ of the aldehyde mixture is needed to

TABLE 2. Response of *Varroa* on a Baudruche Membrane to Mixtures of Synthetic Primary Aliphatic Alcohols and Aldehydes Identified in Active Fractions of Honey Bee Cocoon Extracts

Test material	Quantity on treated area ^b µg/cm ²	N <i>Varroa</i> tested	% reacting <i>Varroa</i> ^a	Fisher's exact test compared ^b to:		Intensity of reaction	
				cocoon	control	Returns ^c mean ± SEM	Tukey's test
Solvent	60 µl	26	4	****		4	/
Cocoon from comb ^d	0.5 cceq	31	77		****	23.9 ± 3.4	a
C ₁₆ +C ₁₈ +C ₂₀ +C ₂₂ +C ₂₄ + C ₂₆ +C ₂₈ +C ₃₀ -OH	18 each	11	64	n.s.	****	21.4 ± 5.7	a
C ₁₆ -OH + C ₁₈ -OH	18 each	22	55	n.s.	***	15.1 ± 2.9	a
C ₁₈ -OH + C ₂₀ -OH	18 each	36	78	n.s.	****	28.4 ± 3.6	a
C ₂₀ -OH + C ₂₂ -OH	18 each	24	75	n.s.	****	25.5 ± 4.3	a
C ₂₄ -OH + C ₂₆ -OH	18 each	25	12	****	n.s.	8.3 ± 2.3	/
C ₂₈ -OH + C ₃₀ -OH	18 each	24	25	***	*	3.5 ± 0.9	b
C ₂₄ +C ₂₆ +C ₂₈ +C ₃₀ -OH	18 each	12	17	***	n.s.	6 ± 2	/
Solvent	60 µl	23	4	****		3	/
Cocoon from comb ^d	0.5 cceq	19	84		****	32.9 ± 4.5	a
C ₁₆ +C ₁₈ +C ₂₀ +C ₂₂ +C ₂₄ + C ₂₆ +C ₂₈ +C ₃₀ -CHO	3 each	33	70	n.s.	****	23 ± 3.5	ac
C ₁₆ +C ₁₈ +C ₂₀ +C ₂₂ -CHO	3 each	12	33	**	*	6 ± 0.9	bc
C ₁₆ +C ₁₈ +C ₂₀ +C ₂₂ -CHO	5.3 each	24	75	n.s.	****	22.2 ± 3.4	abc
C ₂₄ +C ₂₆ +C ₂₈ +C ₃₀ -CHO	3 each	12	0	****	n.s.	/	/
C ₂₄ +C ₂₆ +C ₂₈ +C ₃₀ -CHO	5.3 each	21	38	**	**	7 ± 1.1	b

^aA *Varroa* was considered as "reacting" to the test material if it walked for ≥50 s on the treated area or if it made at least four returns toward the treated area on encountering the border.

^bFisher's exact test: n.s. = non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

^cMean number of returns per run. Only reacting mites are considered when calculating the means ± SEM for the ANOVA and Tukey multiple comparison test. The numbers of returns were transformed (natural logarithm) prior to statistical analysis. For each experimental series, the treatments assigned a different letter differ significantly at $P < 0.05$.

^dOne cceq represents the extract of the cocoon pulled out of one comb cell.

induce a strong arrestment in more than 50% of mites tested, despite the fact that the crude cocoon extract contains only about 0.5 to 0.6 µg/cm² of the behaviorally active primary alcohols and less than 50 ng/cm² of the active aldehydes. Why then should such a high quantity of synthetics be needed to induce *Varroa* to react? One explanation could be a synergism between the different chemical groups in the cocoon extracts. All three binary mixtures of the three active functional groups, i.e., nonadecane and eicosane for the hydrocarbons, octadecanol and eicosanol for the alcohols, and eicosanal and docosanal for the aldehydes tested at a total dose of 12 µg (6 µg each) induced comparable responses, with 57–67% of reacting mites returning 14 ± 2.2 to 19 ± 3.2 times at the treatment borders (Fig. 7). Quaternary mixtures comprised of hydrocarbon + aldehyde pairs and hydrocarbon + alcohol pairs tested on the same day at a total amount of 12 µg (i.e., 3 µg of each compound) elicited a better response than the binary mixtures on their own for both the proportion of reacting mites and the response intensity (Fig. 7). On the other hand, the quaternary mixture of the alcohol +

aldehyde pairs proved inactive for all the five lots of mites tested (Fig. 7 and Table 3; $n = 49$, n.s. compared to control).

Since the quaternary mixture of alcohol + aldehyde pairs at the 1:1 ratio was not active, a solution at a 10:1 proportion (approximating that of cocoon extracts), was tested. Although this 10:1 solution was tested at lower total doses than the 1:1 solution, i.e., 6.6 µg and 33 µg as against 12 µg and 60 µg, respectively, (Table 3) a higher response was obtained with the 10:1 ratio at both doses. The synergistic effect was higher at the lower dose than at the higher dose.

Synthetic Cocoon. Considering the synergistic effects of mixtures of functional groups, we finally wondered if a synthetic cocoon solution could induce an arrestment effect as good as the extract of the cocoons spun on glass (gceq). *Varroa*'s response to a solution containing almost all active hydrocarbons, alcohols, and aldehydes at the approximate proportions found in the cocoon extract (Table 3) was strong when tested at 1.0 gceq on the treated area (i.e., 0.3 gceq/cm²) and elicited arrestment in 76% of mites. By comparison, the extract of cocoons spun on glass elicited a positive

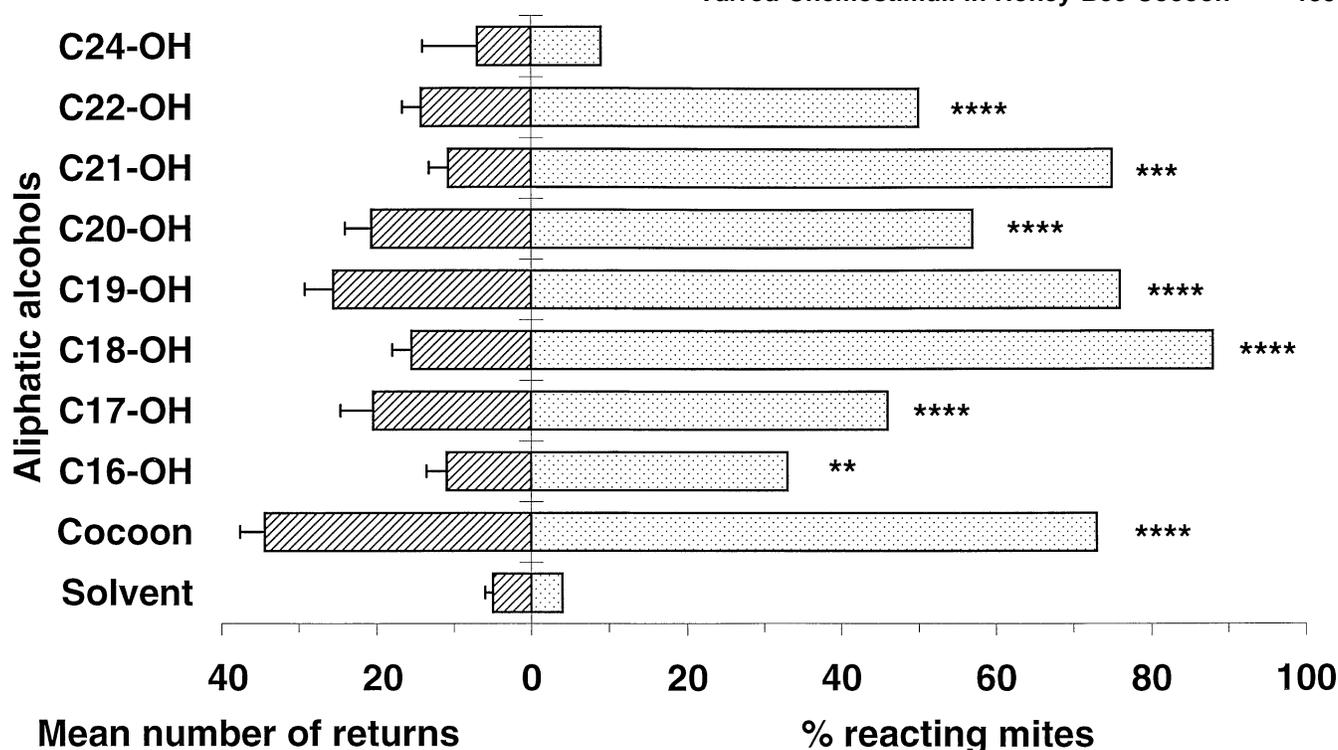


Fig. 4. Behavioral responses of *Varroa* to primary aliphatic alcohols applied singly ($18 \mu\text{g}/\text{cm}^2$) to a semipermeable membrane compared to the solvent (CH_2Cl_2 $60 \mu\text{l}/\text{cm}^2$ before evaporation) and the cocoon extract ($0.5 \text{ cceq}/\text{cm}^2$). The responses were quantified by the percentage of mites ($n > 21$) reacting, and the intensity of their response was characterized by the mean \pm SEM number of returns at the border of

treated area. A mite was considered as reacting when it walked longer than 50 s on the treated area or when it proceeded to make at least four returns at the treatment borders. The percentages of reacting mites were compared to the solvent control; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (Fisher's exact test).

response in 35% of mites. If one considers the high proportion of alkanes in the synthetic cocoon mixture ($8.6 \mu\text{g}/\text{gceq}$), one might be led to conclude that they are active on their own. But these combined alkanes tested at the 1.0 gceq induced a response in only 19% of mites ($n = 21$, n.s. compared to the control). When the cocoon extract spun on glass was compared to the synthetic cocoon solution at the 5.0 gceq , the cocoon extract showed an even stronger arrestment response than the synthetic mixture.

DISCUSSION

Silk production is used as protection for pupae in many insects. The spinning bee larva adds colorless and yellow anal secretions to the silk (reviewed by Jay, 1963) and the presence of fatty acids may indicate the role of these secretions as microbial inhibitors (Bienvenu et al., 1968; Saito and Aoki, 1983). Furthermore, aldehydes act as ant repellents (Eisner et al., 1996) and since they are chemically reactive they could also defend

against microorganisms. In a variety of arthropods, silk is used as a pheromone source for group living and mating (Penman and Cone, 1974; Roesingh, 1990; Evans and Main, 1993; Saito Y, in press). Indeed, chemical cues from silk are also used by predators and parasites for the recognition of their hosts (Bekkaoui and Thibout, 1993; Weseloh, 1977).

Chemosensory Response of *Varroa* to Cocoon Constituents

Varroa shows a strong arrestment response on the semipermeable membrane treated with honey bee cocoon extract. It was not rare to find that the last mite tested on a day was still on the treated area the next morning. The arrestment behavior induced by the cocoon extract was the same as that of bee larval extracts (Rickli et al., 1994). This is characterized by an immediate border recognition and returns on to the treated area without overshoots. In doing so, *Varroa* avoids loss of contact with the treated

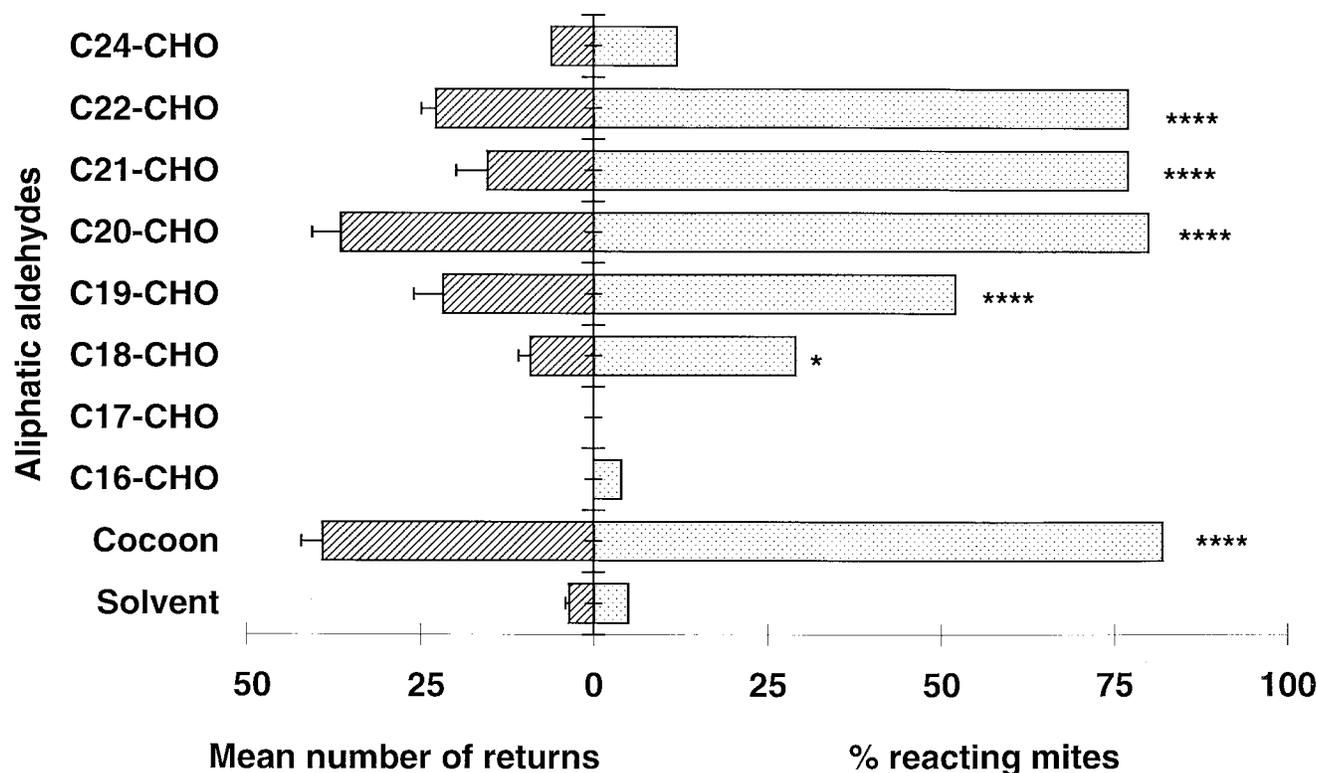


Fig. 5. Behavioral responses of *Varroa* ($n > 20$) to primary aliphatic aldehydes applied singly ($12 \mu\text{g}/\text{cm}^2$) to a semipermeable membrane compared to solvent control (CH_2Cl_2 60

$\mu\text{g}/\text{cm}^2$ before evaporation) and the crude cocoon extract ($0.5 \text{ cceq}/\text{cm}^2$). Quantification of the responses and significant differences were assigned as in Figure 4.

area. Predatory mites display a similar arrestment on filter paper treated with silk and associated feces of their prey *Tetranychus urticae* (Hislop and Prokopy, 1981).

Aliphatic aldehydes C_{18} to C_{22} were identified as active constituents of TLC fraction F_2 but none of the other tested compounds of this fraction induced arrestment. However, the amount of aldehydes in F_2 could not induce the full response observed for this fraction, so it is probable that other compounds synergise the response. In fraction F_4 , primary aliphatic alcohols C_{16} to C_{22} elicited arrestment. As for fraction F_2 , the quantity of active alcohols in F_4 is lower than the threshold observed in the bioassay. The role of primary C_{14} – C_{34} acids in this fraction remains unclear. A mixture of the five saturated C_{18} – C_{22} -COOH each at $18 \mu\text{g}/\text{cm}^2$ elicited a positive response in 68% of mites, but none of these acids elicited a good response when tested alone (max. 25% of reacting mites on $18 \mu\text{g}/\text{cm}^2$ of C_{20} -COOH; Donzé, unreported data). It is not clear if the mixture of fatty acids synergise as observed for alkanes (Rickli et al., 1994; Grenier et al., 1993) or if the response

was due to impurities in the standards. Furthermore, the high threshold needed for the arrestment response with synthetics may have originated from the bioassay conditions. The membrane is somewhat porous, and polar functional groups may have a higher affinity with the underlying water than the hydrocarbons. This could explain why alcohols are less active than aldehydes at low doses.

Rickli et al. (1994) identified *n*-alkanes from bee larvae as arrestants on the same membrane bioassay employed here. It was thus surprising that our least polar TLC fraction F_1 , which contained the saturated alkanes, did not elicit more returns than the solvent control. This difference is probably due to the fact that our fraction contained other substances which inhibited or masked the hydrocarbon activity. Indeed, when the hydrocarbons were isolated from the cocoon extract by column chromatography they did induce clear arrestment. However, a reduction in this response was again observed when both the nonpolar wax ester and hydrocarbon fractions were mixed (Donzé, unreported data).

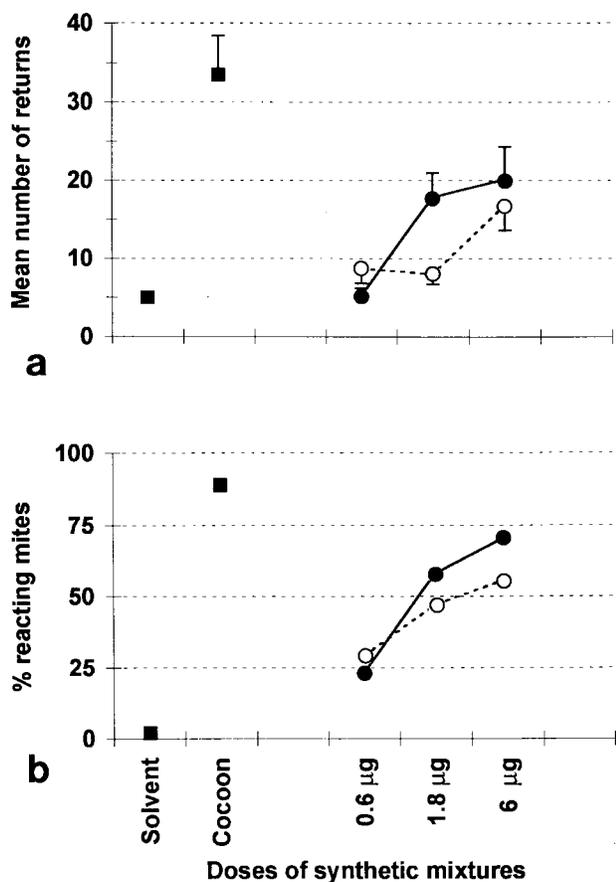


Fig. 6. Responses of *Varroa* to increasing doses of mixtures of the four most active primary alcohols (i.e., C₁₆+C₁₈+C₂₀+C₂₂-OH) and two most active primary aldehydes (i.e., C₂₀+C₂₂-CHO) identified in honey bee cocoon extracts. The mixture was applied to a semipermeable baudruche membrane. (a) Response intensity of the reacting *Varroa* as indicated by the number of returns at the treated zone borders. (b) Percentage of mites walking longer than 50 s on the treated area or proceeding to make at least four returns at the treatment borders (i.e., above the 95% limits of control runs). Squares indicate the response on the solvent (CH₂Cl₂ 80 µl/cm² before evaporation; n = 42 mites), and on the cocoon extract (0.5 cceq/cm²; n = 19 mites). Open circles indicate responses to the alcohol mixture (n > 21 mites), and filled circles to the aldehyde mixture (n > 22 mites) at doses of 0.6, 1.8, and 6.0 µg/cm² of each constituent.

Behavioral Response of *Varroa* to Specific Blends

Primary aldehydes and alcohols which are available in small amounts in the cocoon increased the arrestment response of *Varroa* mites when mixed with higher amounts of straight-chain saturated hydrocarbons. The ability of *Varroa* to respond to specific blends of products was confirmed by an arrestment response which was only elicited at proportions of oxygenated

lipids similar to that occurring in the cocoon. It is noteworthy that the specific inhibition of arrestment was limited to the alcohol + aldehyde mixture and does not occur with alkanes. The optimal response of *Varroa* was obtained with a cocktail of alkanes, aldehydes, and alcohols simulating the cocoon extract. This cocktail induced even a stronger response than the crude extract. Synergism between products of one functional group was already documented for *Varroa* with the alkanes series C_{23:0}-C_{29:0} when bioassayed in binary mixtures, where these products did not elicit arrestment when tested singly (Rickli et al., 1994). This selective response to blends of different functional groups at appropriate proportions is adaptive in the beehive, where aliphatic compounds dominate, occurring in a variety of bee instars and bee products. In honey bees, confronted with the same environment as *Varroa*, evidence for responses to blends has been detected: blends of fatty acid esters differ as a function of caste and age of brood, and worker bees behave differently in the presence of these mixtures (Le Conte et al., 1994, 1995).

Another observation indicates the chemosensory specificity of *Varroa*. Fatty acid esters and palmitic acid have been shown to attract *Varroa* in olfactometers (Le Conte et al., 1989; Rickli et al., 1992) but they did not induce arrestment on the membrane in this study. Therefore, different chemostimuli would appear to be involved in host orientation and host acceptance in *Varroa*. Contact chemostimuli could permit recognition of the substrate on which *Varroa* walks and the volatile chemostimuli could mediate simultaneous olfactory-guided orientation to the target.

We have now established that *Varroa* uses several functional groups as semiochemicals, and most of the active molecules have a chain length of C₁₆ to C₂₂. The fact that these occur in smaller quantities than longer-chain compounds (>C₂₂) may be advantageous. It could be hypothesized that the evolution of this trait arose because the use of the less abundant compounds as chemostimuli by *Varroa* enhances selectivity for host instar recognition. When present in too small a quantity on one instar, the stimuli would not be detected since they are under the detection limit, but could occur over that limit in another instar. By contrast, use of compounds present at high levels throughout the instars would require a high degree of tuning of the

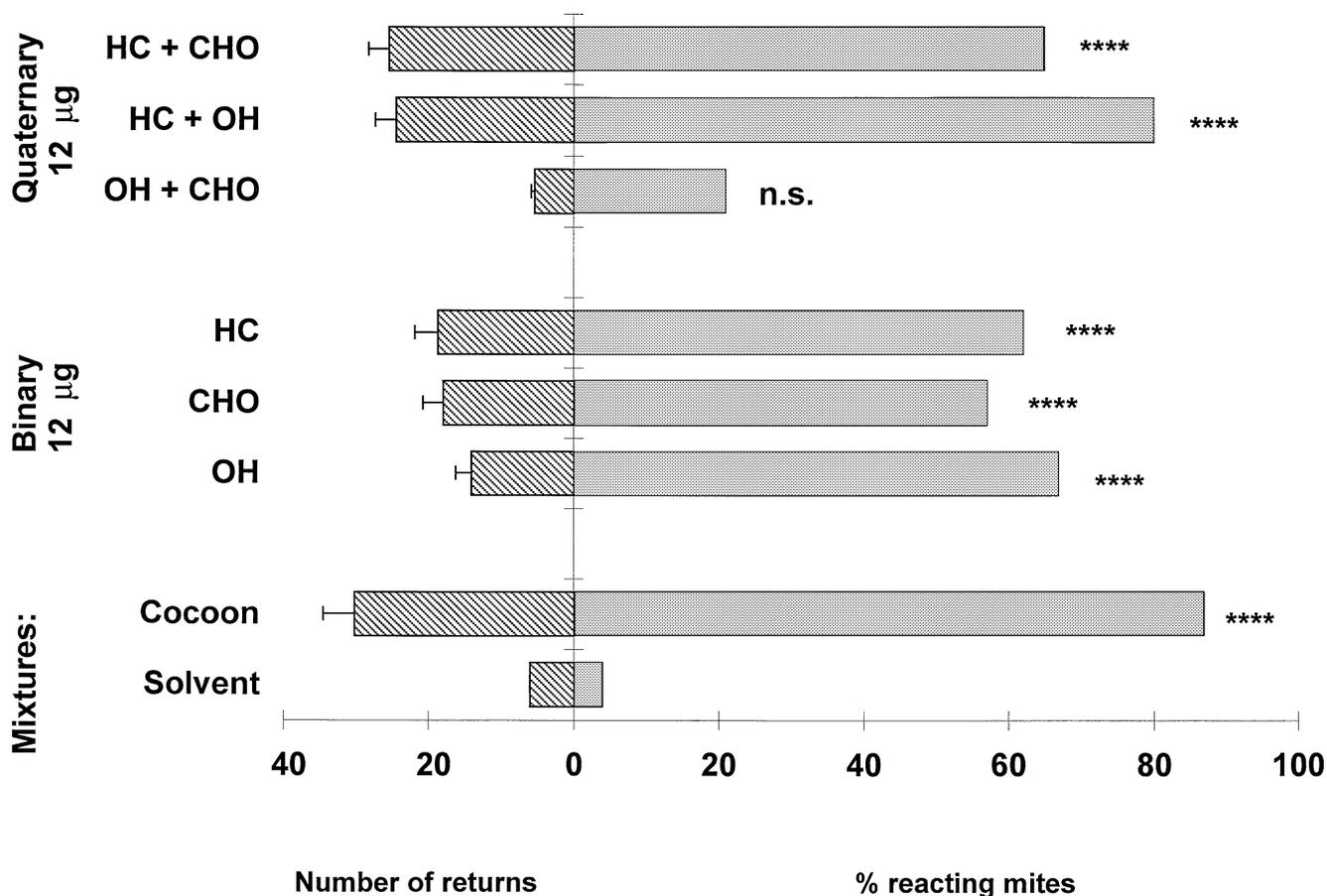


Fig. 7. Response of *Varroa* ($n > 19$) to paired hydrocarbons (HC), i.e., nonadecane + heineicosane, primary alcohols (OH), i.e., octadecanol + eicosanol, and aldehydes (CHO), i.e., eicosanal + docosanal. Each functional group was first tested with a binary mixture of the two active compounds at a total dose of 12 µg (6 µg each). Then the functional groups were paired in quaternary mixtures and tested at a total dose of

12 µg (3 µg each). The cocoon extract was tested at 1.7 cceq's and the control at 100 µl CH_2Cl_2 before evaporation on the treated area (339 mm^2). Quantification of the responses was assigned as in Figure 4. All mixtures were significantly different to the control ($P < 0.0001$) except for the CHO + OH quaternary mixture (Fisher's exact test).

chemosensory system to detect any small differences in the profiles.

Distribution of Chemostimuli Within the Hive

Some oxygenated compounds identified in the cocoon which affect *Varroa*'s behavior also occur in the beeswax and cuticular lipids, and extracts of these substrates induced an arrestment similar to the cocoon extracts. Long-chain alcohols with 24 or more C-atoms associated with the cocoon extracts have been found in comb wax (Tulloch, 1971) but only in small quantities in larval cuticle (this study). Free fatty acids C_{16} – C_{20} are present in bee cuticular lipids but are not accounted for in comb wax (Tulloch, 1971). By using cocoons spun on glass, in this study we excluded contamination of such

material by the underlying wax. Since the prepupae have small quantities of aldehydes and alcohols on their surface, it is not possible to exclude contamination from the cocoon. Nevertheless, the young pupa has a new cuticle which already contains alcohols and aldehydes. This may suggest that the bees produce aliphatic alcohols in most instars and, like the bee cocoon, the major alcohols in the cuticle of insects, including bees, range from C_{22} to C_{34} , whereas alcohols of medium chain length are absent or present only in small quantities (Buckner, 1993; this study). This pattern also seems to be valid for the aldehydes, since preliminary results have shown C_{26} and C_{28} -CHO to be the more abundant aldehydes in the cocoon (Schnyder-Candrian, unreported data). Hydrocarbons follow the same pat-

TABLE 3. Response of *Varroa* on a Baudruche Membrane to Mixtures of Synthetic Primary Aliphatic Alcohols, Aldehydes, and *n*-alkanes Identified in Active Fractions of Honey Bee Cocoon Extracts

Test material	Quantity on treated area ^a	N <i>Varroa</i> tested	% reacting <i>Varroa</i> ^b	Fisher's exact test compared ^c to:		Intensity of reaction	
				cocoon	control	Returns ^d mean ± SEM	Tukey's test
1) Proportions of R-OH: R-CHO ^e							
Solvent	50 µl	15	7	****		6	/
Cocoon from comb ^a	1 cceq	42	83		****	25 ± 2.5	a
C ₁₈ + ₂₀ -OH+C ₂₀ + ₂₂ -CHO	2×3+	30	13	****	n.s.	5.3 ± 0.5	/
1:1 ratio	2×3 µg						
C ₁₈ + ₂₀ -OH+C ₂₀ + ₂₂ -CHO	2×3+	44	57	**	***	9.2 ± 1.4	b
10:1 ratio	2×0.3 µg						
C ₁₈ + ₂₀ -OH+C ₂₀ + ₂₂ -CHO	2×15+	18	56	*	**	23.4 ± 2.4	a
1:1	2×15 µg						
C ₁₈ + ₂₀ -OH+C ₂₀ + ₂₂ -CHO	2×15+	18	78	n.s.	****	26.4 ± 2.4	a
10:1	2×1.5 µg						
C ₁₈ + ₂₀ -OH	12×3 µg	21	19	****	n.s.	6.5 ± 1.3	/
C ₂₀ + ₂₂ -CHO	2×0.3 µg	10	0	****	n.s.	/	/
2) Synthetic cocoon vs. cocoon spun on glass							
Solvent	50 µl	20	0	****		/	/
Cocoon from comb ^a	1 cceq	34	79		****	24 ± 2.5	ac
Synthetic: total ^f	0.2 gceq	21	19	****	n.s.	10.8 ± 2.5	/
Synthetic: total ^f	1.0 gceq	33	76	n.s.	****	25 ± 3.4	ac
Synthetic: total ^f	5.0 gceq	20	80	n.s.	****	21.2 ± 2.6	c
Synthetic: alkanes only ^f	1.0 gceq	21	19	****	n.s.	14 ± 0.8	/
Cocoon spun on glass ^g	1.0 gceq	20	35	**	**	4.3 ± 0.5	b
Cocoon spun on glass ^g	5.0 gceq	20	85	n.s.	****	35.4 ± 2.7	a

^aThe treated area of the membrane measured 339 mm². One cceq represents the extract of the cocoon pulled out of one comb cell.

^bA *Varroa* was considered as "reacting" to the test material if it walked for ≥50 s on the treated area or if it made at least four returns toward the treated area on encountering the border.

^cFisher's exact test: n.s. = non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

^dMean number of returns per run. Only reacting mites are considered when calculating the means ± SEM for the ANOVA and Tukey multiple comparison test. The numbers of returns were transformed (natural logarithm) prior to statistical analysis. For each experimental series, the treatments assigned a different letter differ significantly at $P < 0.05$.

^eR-OH alcohols; R-CHO aldehydes (e.g., C₂₀-CHO = eicosanal).

^fIn accordance with quantification of compounds in extracts of cocoons spun on glass (Fig. 3) a cocktail of synthetics was made up containing: 100 ng C_{19:0}, 500 ng C_{21:0}, 3000 ng C_{23:0}, 5000 ng C_{25:0}, 100 ng C₁₈-CHO, 20 ng C₂₀-CHO, 20 ng C₂₂-CHO, 500 ng C₁₈-OH, 300 ng C₂₀-OH, and 200 ng C₂₂-OH.

^gLarvae at the time of capping were transferred to a crystallizing dish where they applied their cocoons on the glass. One gceq represents the cocoon spun by one larva.

tern, with C_{25:0}-C_{29:0} being the major alkanes in all bee instars (Fig. 3; McDaniel et al., 1984; Nation et al., 1992; Francis et al., 1989).

Chemosensory Ecology of *Varroa*

The question arises how the parasite uses the semiochemicals to differentiate the successive host instars during its lifecycle. After emergence, *Varroa* females leave the young bee to infest middle-aged nurse bees (Kraus, 1993; Steiner, 1993; Kuenen and Calderone, 1997), which are more likely to carry them onto new brood. *Varroa* avoids forager bees, from whom there is more risk of becoming lost outside the hive. Nasonov and sting secretions could be im-

plicated in the avoidance of foragers (Hoppe and Ritter, 1988; Kraus, 1990). We did not find aldehydes on the cuticle of adult bees, and alcohols are present in higher amounts on the cuticle of older bees than on younger ones. Currently, we cannot conclude whether these lipids play a role in the worker bee age recognition by *Varroa*. Once *Varroa* descends from the nurse bee it encounters wax coated with cocoon (Boot et al., 1994). This substrate is drastically different to the bee and could be an important stimulus to help the orientation behavior of the parasite toward the larva. Since aldehydes are absent from older larvae of both drones and workers, and as the active alcohols are present on larvae of both

sexes only in small quantities, these products probably play no role in the recognition of the sex of bee larvae. This recognition is thought to be mediated by the cuticular fatty acid esters (Trouiller et al., 1992, 1994).

Our observations on *Varroa* reproduction in transparent artificial brood cells have shown the importance of the cocoon substrate to deposit feces used as aggregation cues and for egg-laying and molting, while the developing bee is used only for feeding (Donzé and Guerin, 1994, 1997; Donzé et al., 1996). Prior to defecation, oviposition, and molting, the mites perform characteristic searches on the cocoon during which they encounter chemically marked structures. This leads them to select sites which enhance survival. In the present study, we confirm that the cocoon contains semiochemicals for *Varroa*. Since aldehydes and alcohols are present in the cocoon at higher amounts than on the developing bee, they may allow differentiation between such substrates and may be implicated in the spatial behavior of *Varroa* during its reproductive phase. Alkanes could also play a role in spatial behavior since they are present in different quantities in the cocoon than in cuticular lipids of the developing bee. In addition to chemostimuli, geotaxis and proprioceptive stimuli probably act as additional cues leading to the selection of sites for fecal accumulation, egg deposition and molting by nymphs (Donzé and Guerin, 1994, 1997).

Varroa's cuticle is coated with hydrocarbons similar to that of the host instar being parasitized (Nation et al., 1992) and this mimicry may serve to reduce detection of the mite by the bees (Rosenkranz et al., 1993; Boecking and Ritter, 1994). It would be interesting to know if this camouflage also extends to oxygenated compounds since we know that honey bees are able to detect tetradecanol, hexadecanoic acid, and C₉–C₁₁ aldehydes by olfaction (Thierry et al., 1990; Dickens et al., 1986).

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