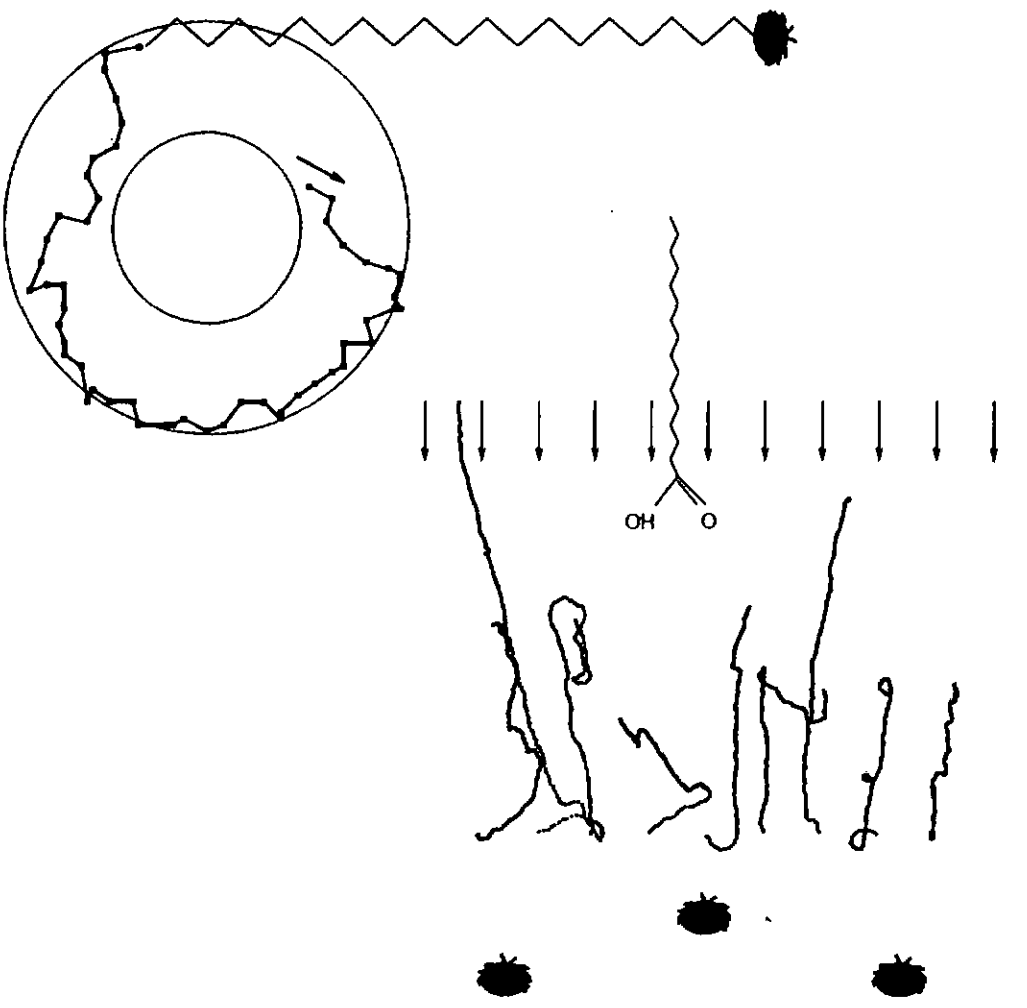
Université Neuchâtel Faculté des Sciences

Chemostimuli involved in host finding and recognition in *Varroa jacobsoni* Oud., a honeybee parasite



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Chemostimuli involved in host finding and recognition in Varroa jacobsoni Oud., a honeybee parasite

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UNIVERSITÉ DE NEUCHÂTEL

FACULTÉ DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel
sur le rapport des membres du jury,

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autorise l'impression de la présente thèse.

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Le doyen :

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Contents

1. SUMMARY

1) The orientation of the ectoparasitic mite *Varroa jacobsoni* towards volatile and contact-chemicals of its host *Apis mellifera*, i.e., odour and taste, respectivaly, was studied *in vitro* in two bioassays. Specific behavioral responses of the mites, i.e., attraction towards an odour source in olfaction bioassays and arrestment in tests for gustatory stimuli, were observed and used to determine the activity for the mites of host extracts, extract fractions and synthetic constituents.

2) *Varroa* responds to volatile compounds released by honeybee worker larvae with walking towards the wind bearing the odour. Thus an anemotactic behavior elicited by the presence of the stimulus was demonstrated. Palmitic acid ($C_{16:0}$ fatty acid) present in acitve odour condensates elicited a similar response.

3) Upon contacting chemostimuli extracted from the cuticle of bee larvae the mites respond with arrestment. Saturated hydrocarbons (HC) ware identified as active compounds. An individual component (*n*-$C_{21:0}$) elicits a similar response as the extract. But a comparison of the activity thresholds of cuticular HC's, synthetic straight-chain HC's and *n*-$C_{21:0}$ demonstrates that mixtures of HC's have synergistic effects on the arrestment response. Compounds found active in olfaction were inactive when the mites could contact them.

4) The response of *Varroa* contacting larva cuticle extract has two components: high walking speed along with straighter paths while walking on the extract and returning back onto the stimulus when touching its borders. The net effect is an arrestment, i.e., the mite stays far longer on an area treated with active material than on a solvent treated substrate.

5) GC-MS analysis showed that active contact-chemostimuli, i.e., saturated HC's are present on the mites themselves. This observation confirms the hypothesis of a chemical mimicry in *Varroa* which may serve to reduce detection by the host. Furthermore, these components may be implicated in the relationship between mites of the same brood cell.

6) The presence of straight-chain alkanes on all honeybee life-stages and in beeswax leaves open as to how *Varroa* recognizes the appropriate host. Two candidate cues for discrimination are named: internally branched alkanes and fatty acid esters. Both stimuli elicited differences in the walking behavior compared to solvent alone, but not border recognition.

2. INTRODUCTION

Varroa jacobsoni Oud. (Acari; Mesostigmata) is an ectoparasitic mite which sucks hemolymph of bees. It reproduces within sealed brood cells of bees of the genus *Apis* (Insecta; Hymenoptera). It was first found on the Asian bee *Apis cerana* Fabr., and it is thought that there has been a co-evolution between parasite and host leading to defensive behavior on the part of the host and evasive reactions in the parasite. The mite was restricted to *A. cerana* (and probably other *Apis*-species in Asia) because of the geographical isolation of *A. cerana* in Asia and *A. mellifera* L. in Europe, where the eastern limit is a line from the Urals to Iran. Once *A. mellifera*-colonies were imported into the *A.cerana* habitat, the mite accepted this new host. Today it has virtually spread world-wide.

In the European honeybee, *A. mellifera*, the natural dispersion from one host colony to the next is related to the breakdown of heavily infested bee hives. Firstly, when honeybee colonies break down honey-robbing occurs and mites change to invading bees (Sakowsky, 1989; Imdorf and Kilchenmann, 1991). Secondly, *Varroa* infested bees whose colonies break down endeavour to access other colonies by offering regurgitated honey to guarding bees at the entrance of the foreign hive. So parasitization of a host colony until it collapses might not be so maladaptive for the parasite as would appear since it may contribute to *Varroa* dispersion. This places *Varroa* close to insect parasitoids on which so much work has been done concerning host selection *sensu* Vinson (1976). Further, drones often change from one colony to the next and, when carrying mites, also serve to spread the parasite. Mites are spread over bigger distances when colonies swarm. Swarming and drone dispersal might be the major means of dispersion for the paresite between *A. cerana* colonies where collapse of hives due to *Varroa* has not been reported.

2.1. Harm inflicted by *Varroa*

When an adult *A. mellifera* is parasitized by a single mite no direct harm is observed. But the parasitization of larvae and pupae has a strong impact on the host. When one female mite invades the brood cell and reproduces within a shortened lifespan is observed in the emerging bee along with a reduction in weight. Reduced development of the hypopharyngeal glands (production of the food jelly for larvae in adult bees) is also reported for the emerging bees (Schneider and Drescher, 1987). Invasion of the brood cell by more than one adult mite leads to greater harm and if more then 3 mites invade the cell and successfully reproduce then a crippled bee with deformed wings and shortened abdomen will often emerge. If too many bees are parasitized as juveniles then the brood goes unfed and is cared for insufficiently, so the colony disintegrates. Additionally, bacterial and viral brood diseases often break out (Ball, 1985; Glinsky and Jarosz, 1992).

A different situation is observed in the case of *A. cerana*, where worker bees remove mites from worker brood cells but not from drone cells (Rath and Drescher, 1990). Furthermore, lower reproductive success is observed when mites are experimentally introduced into worker brood cells than in drone cells (Tewarson et al., 1992). If more than 3 mites reproduce in a drone cell, the host later is too weak to emerge and dies, thus

imprisoning the mites (Rath, 1992). The virulence of *Varroa* is strongly reduced in *A. cerana*.

2.2. *Apis mellifera* life-cycle

Since the parasite's life-cycle is strongly linked to the host's, a description of the bee's development is given here and because I have worked only with stimuli from worker larvae of *A. mellifera* I will concentrate on worker bee development. If we assume that the honey bee queen lays an egg on day 0 in a brood cell then three days later the larva hatches. Four larval instars at moulting intervals of roughly 1 day follow (Winston, 1987). During the fifth larval instar, i.e., on day 9 the brood cell is sealed with a wax cap by worker bees (operculation). The larvae are fed from hatching to operculation (Brouwers et al., 1987). Nurse bees, i.e. bees of 5-12 days after their imaginal moult, deposit the last bout of larval food at the bottom of the cell before operculation. At operculation the larva completely fills the lower part of the cell and weighs 150 mg (Thrasyvoulou and Benton, 1982) to 170 mg (Goetz and Koeniger, 1992). The day before operculation the larva's weight increases by ca. 75 mg (Goetz and Koeniger, 1992 and references therein) permitting use of weight and size of larvae to estimate their age. Both chemical (Le Conte et al., 1990) and mechanical stimuli (Goetz and Koeniger, 1992) are involved in the capping behavior of worker bees. The provision of larval food is consumed by the larva in the first 6 h after operculation (Donzé and Guerin, 1994). The larva then spins a fine coccoon before it stretches and becomes a prepupa for the next 50 h until moulting to the pupal stage. Some 20-21 days after the egg has been laid, the pupa moults into an imago and the adult bee stays for 10-20 h in the brood cell before it chews its way through the wax cap and emerges (Donzé, pers. comm.).

2.3. *Varroa* life-cycle

When bees emerge from their brood cells the mites parasitizing them also leave the cell and only adult female mites survive. They climb onto older bees, choosing preferentially 4-6 day-old nurse bees in laboratory tests (Kraus et al., 1986; Steiner, 1993), and 4-12-d old bees harvested in a hive had higher infestation rates than newly emerged or older bees. LeConte et al. (1987) also report a preference for 5 day-old bees and suggest that the slightly higher body temperature of these nurse bees attracts the mites. Chemostimuli also appear to be involved in this preference since nurse bees killed by freezing were still more attractive than newly emerged bees (Kraus et al., 1986). The mites move mostly into the folds between stemites and/or tergites of abdominal segments II/III on adult bees (Delfinado-Baker et al., 1993).

A high percentage of mites stays for 1 or more days on nurse bees before they invade brood cells (Calis et al., 1990). Mites which hatched first have more time to mature within the brood cell before the bee emerges than later maturing females of the same cell (Donzé and Guerin, 1994). That is, there is a great variability between females found on bees. The time the mites stay on adult worker bees might be determined, among other factors, by their imaginal moult within the cell (earlier or later daughter). Mites enter cells of worker larvae during the last 20 h before operculation and drone cells 20-40 h before sealing (Boot et al., 1992b). The mites slip between the larval body and the cell wall to the cell base and bury

themselves in the larval food jelly. When the worker larva consumes the remaining food just after the operculation the mite climbs onto the bee (Donzé and Guerin, 1994). Some 60-70 h post operculation the *Varroa* mother lays the first egg and further eggs follow at 30-h intervals. The first egg is haploid providing the only male (per mother) while the other eggs are diploid which develop into female mites (Rehm and Ritter, 1989). Development is typical acarine from protonymph through deutonymph to adult (Ifantidis 1983). Female adult mites are mated several times after their final moult by their brother and leave the brood cell together with their host (Donzé and Guerin, 1994). Female mites which have not terminated their development and males die and are most probably removed from the hive. Adult females climb onto nurse bees and the cycle starts again.

2.4. Chemoorientation in *Varroa*

The life-cycle of *Varroa* can be separated into that within the sealed brood cell and that outside the cell on nurse-bees. In both cases chemoorientation most probably controls mite behavior to some extent:

- Within the cell *Varroa* initially shows a strong attachement to the larva, staying almost exclusively on the larval body while the host spins the cocoon shortly after the operculation (Donzé and Guerin, 1994). Later on the mite rests in the vicinity of an accumulation of its faeces which it deposits on the cell wall, and establishes the specific feeding site on the abdomen of the pupa.
- When the mites emerge from the brood cell they choose nurse bees for the time spent on adult bees (Kraus et al., 1986; LeConte et al., 1987; Steiner, 1993). Later, they leave the adult bees and invade brood cells occupied by either 8 day-old worker or 9 day-old drone larvae (Boot et al., 1992). Further, drone brood is preferred over worker brood (Schulz, 1984).

Varroa chemoorientation has been studied outside the cell to investigate the preference for nurse-bees (Kraus et al., 1986) and for stimuli released from or present on larvae before operculation (LeConte et al., 1989; Rosenkranz, 1990). The reason for this interest in chemostimuli is probably due to the belief that outside the brood cell mites could be exposed to behavior-modifying chemicals which might contribute to control Varroatosis. Evidence exists that three fatty acid esters (methyl palmitate, methyl linolenate and ethyl palmitate) which are released by larvae attract mites in an olfactometer (LeConte et al., 1989). These compounds - two of them also serving to trigger the capping behavior of nurse bees (LeConte et al., 1990) - are present in increased amounts prior to operculation on the bee larva's cuticle and their levels decrease after operculation (Trouiller et al., 1991).

Studies on chemoorientation differentiate between olfactory and gustatory stimuli. The perception of stimuli is in arthropods basically the same for both modalities, i.e., the molecules penetrate through pore(s) into the lumen of a sensillum where they evoke a change in the polarity at the membrane of the dendrite(s) innervating the sensillum. The molecules of olfactory stimuli are volatile, perceived as an odour. Gustatory chemostimuli are essentially solid and perceived upon contact between the sensillum and the compound, hence the term contact-chemostimulus.

In the study on chemostimuli presented here special focus was placed on the manner of harvesting the stimulus and testing activity on the mite. Odours were sampled from the headspace over 8 day-old worker larvae and tested in an olfactometer. Cuticular washes of bee larvae were reapplied to a substrate and tested as possible contact-chemostimuli. The aim of the study was to gain basic knowledge on the chemoorientation of *Varroa in vitro*, since such behavioral experiments could not be made in beehives.

The main questions were the following:

- Can *Varroa* respond to volatile or contact-chemostimuli from bees *in vitro* ?

- Are these responses associated with specific bee extracts (isolation of active material) which serve as semiochemicals (compounds of information value for the receiver) ? If yes, to which constituents do the mites respond (identification) ?

A publication and a manuscript submitted for publication essentially contain the results of this study. The publication deals with the responses of *Varroa* to palmitic acid, an attractant identified in the headvolume over 8 day-old worker larvae. The manuscript reports on the isolation and identification of a set of saturated hydrocarbons which act as contact-chemostimuli on worker larvae and to which the mites respond by arrestment on the treated zone of an arena. Further, the responses of *Varroa* to the contact-chemostimuli were analyzed in details by using track analysis. Part of this work is reported in the manuscript, and the rest is presented here. Some accompanying unpublished experiments are also described here.

3. MATERIALS AND METHODS

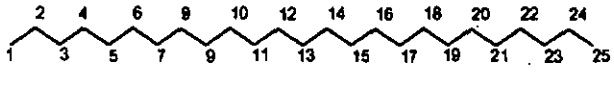
Two main bioassays were devaloped in this study. One for *Varroa* olfaction with the possible volatile chemostimuli delivered to the mites in an airflow. The other for contact-chemicals where the substrate was coated with the possible contact-chemostimulants. Both bioassays are fully described in the respective publications (see chapters 4.1. and 4.2.) along with the treatments of the stimulants and data analysis. Because the publications are placed in the section of results the principle methods are summed up here. Furthermore, some experiments which are not treated in the publications and investigations on the fertility of mites used in bioassays and on the cuticular hydrocarbons of both, host and parasite are described here.

3.1. ANIMALS

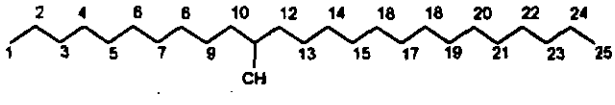
3.1.1. *Varroa*

All mites used in bioassays were harvested in lots of 60 to 100 visible on the surface of adult host bees within heavily infested beehivas of *A. mellifera* (race most probably *A. m. carnica*, but no controlled breeding) at the Department of Apiculture, Swiss Federal Research Station at Liebefeld, Switzerland. Mites usually are found in the intersegmental folds of adult bees and may stay there for an indefinite length of time. By using mites on the surface of the host the category of test-animals was reduced aither to mites freshly emerged from the brood cells or to mites ready for cell invasion. (That such mites are capable of reproduction and consequently may be considered

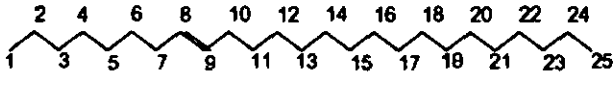
Abbreviations used to describe compounds



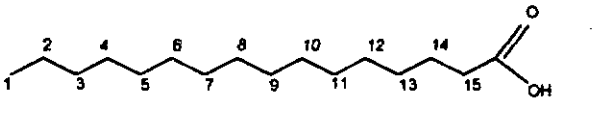
straight chain alkane (n-alkane) with 25 C-atoms, no double bonds (n-C25:0)



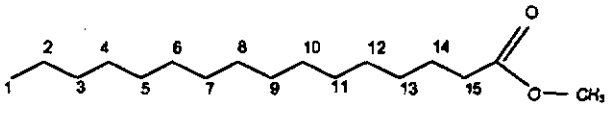
internally branched alkane (br-alkane) with 25 C-atoms and a methyl group added (br-C25:0). Monomethyl alkanes are most abundant, but also dimethyl alkanes were found in extracts analyzed in this study.



straight chain alkene with 25 C-atoms, one double bond (n-C25:1).



Palmitic acid: straight chain with 16 C-atoms, at one end a carboxyl group.



Methyl palmitate (methyl ester of palmitic acid).

as candidates for cell invasion has been shown in the experiment "Fertility of the mites used in bioassays", see chapter 4.4.10.).

The mites were held for 1-7 days on their host in the laboratory after harvesting. At the time of the bioassay a high proportion of the harvested mites were again found in the intersegmental folds of the bees and were detached from there for bioassay.

3.1.2. *Apis mellifera*

Source of stimuli were 8 day-old honeybee worker larvae using their size and weight (> 120 mg, Thrasyvoulou and Benton, 1982) as an index of age. They were harvested from their brood cells immediately before the procedures designed to harvest the chemostimuli in question, i.e., extraction of odours (cold-trap) or extraction of surface compounds (cuticle washes). Only intact worker larvae were used as stimulus sources.

3.2. Collection of chemostimuli

3.2.1. Odours

Volatiles from larvae were cold-trapped by sucking clean air through a glass flask containing 8 day-old worker larvae and through a glass U-tube which was cooled to -70° C. (details see chapter 4.1.). Larval odours condensated on the cold walls of the U-tube, were collected in solvent and concentrated.

3.2.2. Cuticle products from honeybee larvae and their fractionation

Lots of 50 to 200 8 day-old worker larvae were immersed in *n*-hexane (details in chapter 4.2.). Hexane-soluble compounds thus were extracted, the extract then 10 times concentrated under a gentle flow of N_2. Separation of these raw cuticle extracts by thin layer chromatography (TLC) followed by bioassays to determine the behavioral activity of the fractions was made in order to identify the compounds responsible for the mite arrestment. TLC fractions F1 to F9 resulted from the first separation step and the most apolar active TLC fraction F1 was further separated giving the TLC subfractions F1A to F1D (full description in chapter 4.2.). Saturated were separated from unsaturated HC's on argentation TLC, the increased polarity of the solid phase holding back the unsaturated HC's. The *br*-alkanes were separated from the *n*-alkanes by adsorption of the straight chains to a molecular sieve. The nonedsorbed branched chains were recovered from the solvent (iso-octane).

3.2.3. Surface products of host, parasite and comb wax

Each 10 newly emerged workers (≤ 24 h) and hive bees of different ages sampled within the broodnest in the hive were killed by freezing and then extracted for 15 min in 10 ml hexane (analytical grade) along with some 50 mites found on the same bees. Further, to gain knowledge on the ontogeny of the honeybee cuticle HC's profile, 8 day-old worker larvae and 6 to 7 day-old pupae (n=10) were extracted as above (Table 1). The extracts were concentrated under N2 and subjected to GC-

MS analysis (see chapter 3.3.1.). In addition, the walls of brood cells, from which 8 day-old larvae were removed, were rinsed 3 times with 0.5 ml *n*-hexane and the washes were concentrated in order to identify its major components by GC-FID and to confirm - or reject - the presence of known constituents of beeswax (e.g. Tulloch, 1980). A second extraction of beeswax was made by filling 50 empty brood cells with hexane and recovering the solution 15 min later. This extract was highly concentrated and used in bioassays to determine its biological activity and in preliminary GC-FID runs for identification of constituents.

3.3. Identification and quantification

3.3.1. Gas-chromatography coupled with mass-spectrometry (GC-MS)

The contents of cold-trapped larval volatiles were analyzed by GC-MS (conditions see chapter 4.1.) as were the constituents of cuticle extracts of 8 day-old worker larvae and fractions thereof applied to the zoned membrane (see chapter 4.2.). In addition, raw cuticle extracts of 6 to 7 day-old worker pupae, of freshly emerged worker bees, of hive bees of different ages collected in the broodnest, and of *Varroa* harvested from the two categories of adult bees were subjected to GC-MS. The GC conditions were as described in chapters 4.1. ex-

***Table 1:** Hexane extracts of surface products made: sources, chemical analysis (gas-chromatography coupled either with a mass selective detector MS or a flame ionisation detector FID) and bioassays.*

Source of extract	Time of extraction	Detector	Bioassay	Remarks
8 day-old worker larvae	15 min	MS	yes	Identification (Ident.), ontogeny of HC's profile
8 day-old worker larvae	15 min	FID	yes	Quantification
6 to 7 day-old pupae	15 min	MS	no	Ident., ontogeny of HC profile
newly emerged adult bees	15 min	MS	no	Ident., ontogeny of HC profile, comparison with HC's of *Varroa*
worker bees of different ages	15 min	MS	yes	Ident., ontogeny of HC profile, comparison with HC's of *Varroa*
Varroa from newly emerged bees	15 min	MS	no	Ident., comparison with HC's of hosts
Varroa from worker bees of different ages	15 min	MS	no	Ident., comparison with HC's of hosts
wax from brood nest	15 min	(FID)	yes	(preliminary analyses)
walls of brood cells occupied by 8 day-old larvae	3 times rinsed with 0.5 ml solvent	FID	no	Ident., comparison with larvae (cues for host recognition ?)

cept for the oven temperature which was programmed to start for honeybee larvae extract at 60°, for pupa extract at 70° and for adult bee and for *Varroa* extract at 100°C.

3.3.2. Gas-chromatography coupled with flame ionization detector (GC-FID)

To estimate quantities of identified products present in cuticle extracts of honeybee larvae GC-FID was employed (conditions see chapter 4.2.). In addition, the walls of brood cells from which 8 day-old larvae were removed had been rinsed and the washes analyzed in GC-FID as above. Quantification of the major compounds, i.e., ≥ 1% of raw extracts was made on 5 separate extraction (see chapter 4.2.). Extract fractions plus a known amount of internal standard *n*-$C_{24:0}$ (30 to 50 ppm) also were analyzed in GC-FID for two reasons. Firstly, for monitoring the separation quality and, secondly, to keep track on what amounts ware applied to the zoned membrane.

3.4. Bioassays

3.4.1. Tests for volatile stimuli - servoaphere

Odours were delivered to *Varroa* on a locomotion compensator. Because the mites' locomotion was compensated by the servosphere the parasites always walked on the north pole of the sphere, the location to which odours were delivered (details see chapter 4.1.). An airstream bore, alternatively, one of two airflows: one passing through an empty glass flask (control), the other passing through a similar flask containing the odour source. The compensation of the mite locomotion required the recognition of the animal movement. This recognition was used to obtain x, y coordinates per time interval of the mite's position in either of the two airflows. These x, y coordinates were sampled and fed into a computer for track record analysis.

3.4.2. Orientation of *Varroa* in absence of volatile chemostimuli on the servoaphere

Here I report on some aspects of the mites' purely anemotactic behavior (orientation in absence of semiochemicals) which is the basic mechanism of the mites' responses to airborne chemical stimuli. The walking behavior in constant wind conditions and in changes from one to a second windspeed was studied. Furthermore, *Varroa*'s walking behavior in a constant airflow devoid of chemostimuli of 0.2 m s^{-1} (total path recording time 2 min, n = 10 mites; see chapter 4.1.) and in < 0.05 m s^{-1} airspeed of the climatized background airflow alone was recorded (total path recording time 2 min, n = 14 mites) in order to determine the spontaneous upwind turning rate per 10 s track segment.

A positive response to an odour was scored if a mite walking in angles ≥ 60° relative to wind (due upwind 0°) during control changed to angles ≤ 30° during test (see chapter 4.1.). However, this positive upwind turning response could occur spontaneously or could be due to the switch between the two airflows alone, independent of their chemical properties. Therefore the mites' upwind turning behavior was observed when switching either between two airflows of identical speeds of 0.2 m s^{-1} or between different speeds, i.e. from < 0.05 to 0.1 and to 0.2 m s^{-1}.

3.4.3. Tests for contact-chemostimuli - zoned membrane

The cuticle of worker larvae at 20 h before the cells are sealed with a wax cap (see 3.1.2.) was washed in solvent and the resulting extract applied to a biological membrane (Fig.1, for details see chapter 4.2.). Mites were deposited in the center of the test arena and the test runs recorded on video from above. The walking behavior was analyzed to determine behavioral activities of material applied.

3.4.4. Odours, extracts, fractions and compounds bioassayed

3.4.4.a. On the servosphere

Odours of the three beehive components adult bees, 8 day-old larvae and wax from brood combs were tested for their behavioral activity (see chapter 4.1.). Cold-trapped condensates of live larvae then were applied to filter paper and tested against controls with solvent alone applied to the filter paper. In both, test and control the airflow was humidified. The compounds squalene and palmitic acid (PA) found in these condensates (see chapter 4.1.) were dissolved in dichloromethane, applied to filter paper in different amounts and tested. In addition, methyl palmitate (MP) was tested for comparison reasons.

3.4.4.b On the zoned membrane

All fractions obtained by the separation steps were applied to the zoned membrane and tested for biological activity. These were raw hexane cuticle extract from 8 day-old larvae; 8 visible fractions and an empty stripe (TLC procedure control) from

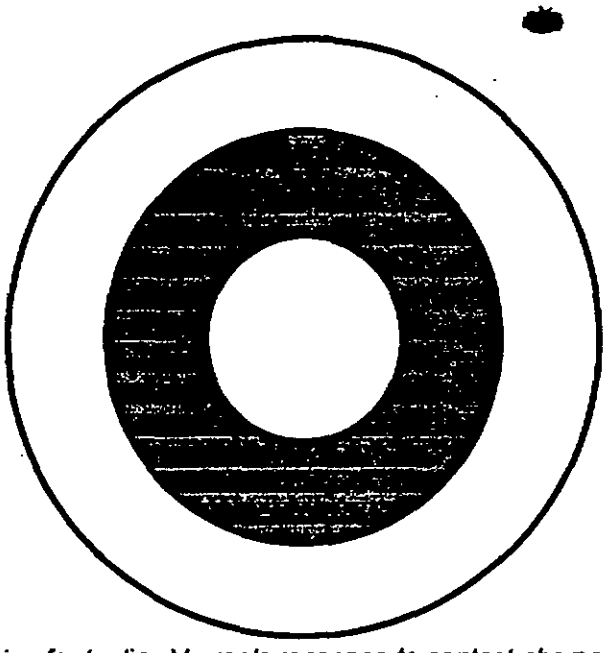


Figure 1: Experimental design for testing Varroa's *response to contect-chemostimuli. On the underside of a semi-permeable biological membrane three circles (diameter 12, 24 and 36 mm) ware drawn with a fine 0.1 mm wide ink pen. The membrane then was washed in acetone and hexane and, efter drying, stretched over a water bath (maintaining test conditions of ≥ 90% r.h. and 32°C). The stimulus was applied to the middle ring (shaded).*

TLC separation (fractions F1 to F9; for details of separation method and amounts applied see chapter 4.2.); the most apolar material (F1) further purified (subfractions F1A to F1D); saturated and unsaturated HC's obtained by argentation TLC; *br*-alkanes recovered after adsorption of the straight chains to a molecular sieve. In addition, extracts made from adult hive bees (n = 50 bees, 15 min extraction) and of wax from a brood comb (n = 50 cells, 15 min extraction) were assayed at amounts of 0.29 bee equivalents and 0.15 cell equivalents per cm^2, respectively.

Synthetic alkenes and *n*-alkanes were dissolved in solvent and tested either singly or in binary or ternary combinations to further identify active compounds out of a set of products present in the active cuticle fractions (details see chapter 4.2). PA was tested in an amount of 10 µg and MP at 5 µg. Further, dose-response relations were established for the TLC-fraction F1A (purified HC subfraction of TLC fraction F1), synthetic *n*-$C_{21:0}$ and a mixture of synthetic *n*-alkanes imitating the proportions found in cuticle extract.

Preliminary experiments also included the application of each 12 larva equivalents per cm^2 of raw hexane cuticle extract to glass (Petri dish) and to filter paper which both were zoned as described for the membranes (see chapter 4.2.). These treated substrates were bioassayed under similar conditions of 32°C and ≥ 90% r.h.. Furthermore, larval extracts with the solvents methanol and dichloromethane were made by immersion of lots of larvae for 15 min in the solvent followed by concentration under N_2 and bioassays.

3.4.5. Fertility of the mites used in bioassays

The intention of this study was to study mites which were about to invade brood cells. In order to enter a cell the mites must leave the adult bees on which they had spent some time (Boot et al., 1992b), i.e., must leave the intersegmental folds first. The mites used in all tests described here had been harvested when visible on the thoracic or abdominal surface of an adult bee within the hive. These mites were then either ready to leave a bee to invade a brood cell, or they had just left a brood cell of an emerging bee. To verify one aspect of this assumption the fertility of mites which were harvested either on the surface of adult bees in the hive (random age) or from newly emerged workers was determined by introducing them into freshly sealed worker brood cells (< 12 h after operculation). Brood cells which were sealed within a patch of 8 day-old worker larvae were marked on a transparent sheet. Between 6 and 10 h later the same comb was checked for cells which had been sealed in the meantime and these cells marked on the same transparent sheet. A small opening was cut at the edge of the wax seal and the mite inserted into such recently sealed cells. The opening was carefully closed with a small amount of melted combwax and the combs returned to the hives. The cells were opened some 6-7 days later and checked for the presence of immature mites. The proportion of cells containing such immature stages which could have terminated their development before bee emergence cell was calculated using the criteria of Fuchs and Langenbach (1989).

3.5. Data analysis

3.5.1. Track analysis on the servosphere

Tracks of 10 s duration in an airflow passing through a control glass flask (either empty or containing a solvent treated filter paper) followed immediately by 10 s tracks in an airstream passing through a flask containing the odour source were recorded. A positive response was defined as an upwind turning mite whose vector angle, i.e., the angle between the wind direction and the straight line joining the start and finish points of a track, shifted from across- or downwind ($\geq 60^{\circ}$ on either side of due upwind) during control to an angle $\leq 30^{\circ}$ during test (details in chapter 4.1.).

To study the attraction of *Varroa* to PA in more detail, tracks of 10 mites were recorded for 60 s in a humidified airstream alone and in combination with the attractant PA. Vector length (mm), deviation angle from wind direction (0°) and the turn angle (difference in walking direction between successive segments of the track) were calculated per 0.2 s interval. The frequency distributions of these track parameters in humidified air alone were compared with those recorded in the presence of PA.

The 2 min track records in airflows devoid of chemostimuli and of 0.2 and ≤ 0.05 m s^{-1} windspeed were cut into successive segments of 10 s to determine the rate of spontaneous upwind turning under constant wind conditions. The number of segments with upwind vectors (vector angles < 30° on either side of the wind direction) following segments where the mites walked across- or downwind (vector angles > 60°) was noted.

3.5.2. Track analysis on the zoned membrane

Analysis of the video recordings of *Varroa* on different doses of a fraction of host cuticle extract and on solvent only was made in order to study in detail the arrestment response observed on membranes coated with cuticular extract. All tests runs used in this track analysis were done on the same day and with mites of the same lot in order to avoid any bias due to day-to-day variations in mite behavior when comparing different doses of larval extract. The runs chosen for examination here are representative of mite behavior as observed in several hundred assays on solvent controls and active extracts in the bioassay system. I chose 10 test runs each on stimulus densities corresponding to 0 (solvent control), 0.6, 1.2, 2.9 and 5.9 larval equivalents (leq) of the nonpolar TLC subfraction F1A (see chapter 4.2.) containing alkanes and alkenes. Tracks of each 10 mites from a different lot on 30 µg *n*-$C_{21:0}$ and on solvent also were analyzed but only to that extent as described in chapter 4.2.

The method to record the basic elements (x, y coordinates in 0.2 s intervals of the pedipalps' position of a test mite, together with the information whether the animal was at each interval fully on the treated area, its borders or outside of the treated area) is described in chapter 4.2. From these basic elements the general track parameters (distance covered and turn angles per 0.2 s, Fig. 3; walking speed and angular velocity per segment from one border contact to the next) were calculated as described there and only the methods used for further analysis are reported here. These additional analyses still employed the x, y coordinates per 0.2 s interval (plus the information "on

the treated area", "on its borders" or "outside of the treated area").

The analysis was applied to track segments where the mites walked either fully on the treated area or in contact with the border. (Tracks from the release point in the centre of the arena to first contact with the treated area and tracks after leaving the extract are not dealt with because they reflect quite another situation for the mites.) First, once the mite was on the treated area, the frequency of 4 types of border contacts was established. Did the mite stay in contact with the stimulus leading it back onto the treated area (called "return"), did it leave the treated area (for at least 0.2 s, called "leaving"), did it stop at the border, or did it move continuously ? Further, the duration of the border contacts as well as the total distance walked and the net displacement along the border (straight line between the first and the last coordinates on the border) was noted.

3.5.3. Analysis of *Varroa* behavior at the borders of an area treated with a contact chemostimulant

The mites seem to walk centrifugally on the zoned membrane from the start of the run on controls, whereas on the stimulus they move rather in circles parallel to the borders on the treated area (Fig. 1 in chapter 4.2 and Fig. 15 in chapter 5.4.3). Returns made at the borders of a treated area allow the mites to regain the treated area and therefore are fundamental to the arrestment response observed. To study in detail what the mite does after a border contact, the key event in maintaining contact with the stimulus, I analyzed a) the arrival angle at the border and b) angles of departure from the border. The basic elements of this analysis are the x,y coordinates which describe the position of the mites' pedipalps at successive 0.2 s intervals. An index was added to indicate whether the mites were fully on the treated area, with either one P1, both P1 or the palps on the border of the

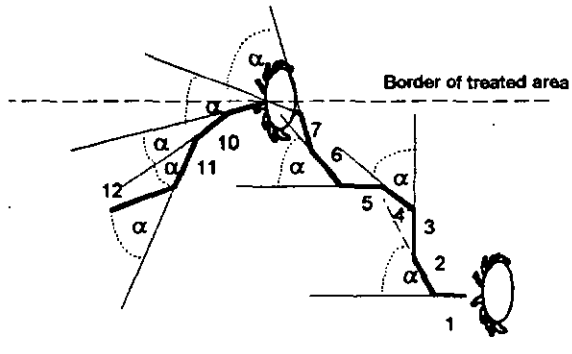


Figure 2: Schema of a segment of path showing the turn angle (α) per 0.2 s interval (numbered 1 to 12). In intervals 7 to 9 the mite is in contact with the border. In reality the mite did not contact a straight but either a convex or a concave border (inner or outer border of the treated ring). Also, the border may be considered as a zone where the concentration of the material applied dropped "steeply" to 0.

treated area, or outside of the treated area (for details see chapter 4.2). Once the mite was on the treated area, I analyzed a) the arrival angle at the border by summing the 0.2 s vector at contact with the border and the two preceding vectors to give a 0.6 s arrival vector (Fig. 2, intervals 5 to 7), and b) angles of departure from the border after a return onto the treated area by summing the last vector on the border and its two subsequent vectors to give a 0.6 s departure vector (Fig. 2, intervals 10 to 12). These longer 0.6 s vectors reflect more accurately the general direction towards and away from the border than the running means of turn angles associated with the shorter 0.2 s vectors which were used in the general description of the walking behavior. Further, in using 0.6 s intervals with distances of 1.5 to 2 mm covered by the mite, the relative importance of pinpointing the exact location of the border contact on the underlying 0.1 mm wide ink circle was reduced. The angle to the tangent at the point of arrival and departure from the border was calculated as the difference between the mite's displacement direction, i.e., that of the 0.6 s arrival or departure vector, and the tangent's own angle in the coordinate system. The latter was defined as the angle of the radius from the coordinates of the point of arrival or departure on the border to the centre of the coordinate system plus 90°. To avoid ascribing arrival and departure angles greater than 90°, adjustments were made by adding ±180° or ±360° to the angle to the tangent depending on which quadrant the mite was walking. The angle to tangent values in degrees thus describe the angular distance between the lines connecting the mite's position 0.6 s before and after the mite's contact to the tangent at the point(s) of border contact. A double check with tracks drawn on a transparent sheet on the video screen gave a close (i.e. within 10°) fit with the values of the angles to the tangent calculated as described above.

It should be noted, however, that a change in the direction of displacement of the mite's palps does not necessarily correspond to the rotation of the mite's body axis. Frame by frame analysis of the video records revealed that mites may arrive at the border, recoil a fraction of 0.34 mm (distance unit on x,y grid) in the next frame and then move sideways over some frames before rotating the body axis to return from the border onto the treated area. Since the position of the waving P1 could not be seen precisely enough in a single frame but well enough in the moving video image over a 0.2 s interval, we refrained from any quantification based on frame by frame analysis. Moving sideways without alignment of the body axis to the displacement direction of the palps for more than 3 frames was observed in only 5 of 133 arbitrarily chosen border contacts on 5.9 leq. Thus detailed movement sequences observed in frame by frame analysis were smoothed out in the 0.2 s intervals.

To provide additional information of what may theoretically happen at the border, an empirical model was made. The picture of a walking mite similar to that shown in Fig. 3, was copied onto a transparent sheet and, based on observations, the centre of gravity of the animal around which the mite turns was assumed to be in the middle of the three leg pairs used for locomotion (pairs II to IV, Fig. 3). The longitudinal body axis (from anus to hypostome) was lengthened beyond the animals dimensions to form a line indicating the direction of

straight forward displacement. An imaginary straight border and arrival directions of 10° each from 0 to 90° were drawn on a sheet of paper. The drawing of the mite was laid over this paper in such a way that the lengthened longitudinal body axis coincided with one of the arrival directions. The "mite" was moved along the arrival direction towards the border until either one P1 or both palps were on the border and then rotated around a needle stuck through the "animal's" centre of gravity. The rotation angles around that centre of gravity required to re-establish full contact with the treated area after hitting a straight border with either one P1 or both palps were measured with respect to the arrival angle (Fig. 3).

To be capable to differentiate between vector directions when the mite was arriving at the border from those when departing, the latter were arbitrarily defined as negative (minus sign in front of angle). This posed some problems in the context of the model (see above) and is discussed in the results section 4.4.8.

The sum of arrival plus departure angles to the tangent at the border (absolute values) represents the rotation in the direction of displacement from arriving to departing (correction angle). The size of arrival, departure and correction angles of returns of 0 to 0.2 s on 1.2, 2.9 and 5.9 leq of TLC subfraction F1A were analyzed by linear regression and Pearson's correlation as were returns after a longer duration on the border (with a maximal displacement of 3.4 mm). Since a rotation around the centre of gravity was measured in the model this means that a mite should pivot to produce a similar movement. For this reason only returns from the border after contacts of 0.2 s or less were used to judge the fit between model and observation.

As the ring of treated substrate has an inner and an outer border (Fig. 1) the correction angles for inner and outer border contacts were correlated separately with the respective arrival angles by linear regression (Pearson's correlation) for 1.2 to 5.9 leq.

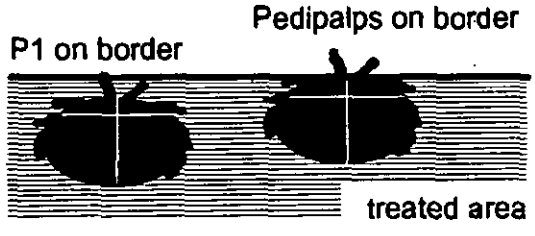


Figure 3: *Two extreme types of border contacts which were used in a model to demonstrate the range of departure and correction angles required to re-establish full contact with the chemostimulant after contacting the border. Mites made contact at a straight border either with one P1 alone (left) or with both its palps (right) before returning onto the treated area. In reality the mites do not contact a straight border but either a convex one (inner) or a concave one (outer). The white cross drawn on Varroa's dorsal sclerite indicates the centre of gravity around which the animal shifts its body axis in this model.*

3.5.4. Determination of behavioral activities on zoned membranes

Test runs were recorded on video from above and the walking behavior was analyzed (details in chapter 4.2.). Data from runs on solvent (control) were pooled and used to define a standard walking behavior on the membrane. If an animal showed walking behavior differing from the standard behavior on solvent controls it was considered to respond to the solution applied. The number of animals showing a reaction to a test solution was compared to the number on solvent controls.

3.6. Statistics

Statistical tests were all made using Systat® version 5.0 (Systat INC., Evanston, Illinois, USA) PC software except where noted. Fisher's exact tests were applied to compare pairwise the numbers of mites showing a specified behavior - or not - in tests to the number of mites in controls or other tests (chapters 4.1., 4.2., 4.4.1., 4.4.2., 4.4.3.c, 4.4.3.d). A Kolmogorov-Smirnov test was used to compare two distributions (chapter 4.1.). Mann-Whitney tests were applied to compare the shapes (mean and standard deviation) of normal distributions for 0.2 s relative turn angles, distances covered per 0.2 s and path straightness per run (chapter 4.1., made on BDMP software package). *Post hoc* ANOVA tests (Tukey-HSD) allowed the determination of differences in normal distributed parameters such as distances covered per 0.2 s, walking speeds and angular velocities per path segment (chapter 4.2.), path straightness, total and vector lengths per path segment (chapter 4.4.2.) or in normalized distributions of absolute turn angles (log of running means over triad of consecutive relative 0.2 s turn angles) in chapters 4.2. and 4.4.2.. In addition, notched box plots on durations of mite behavior (in which 95% confidence intervals of median values are calculated and visualized, Fig. 4, McGill et al., 1978) supported statistical results in analyses per individuals in chapter 4.2.. Cases of non-convergence of the two statistical methods hinted to further but less obvious differences in the mite behavior observed on compared test material.

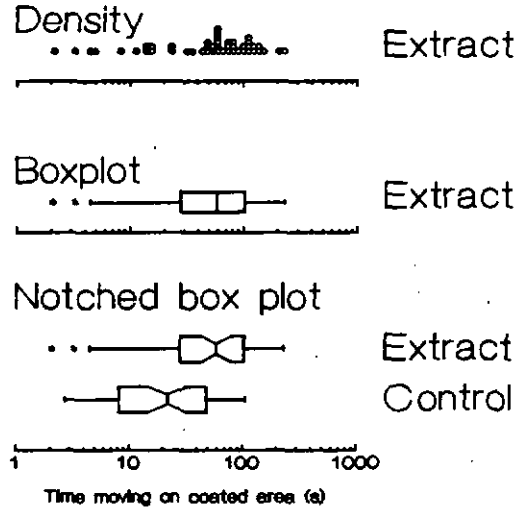


Figure 4: Demonstration of notched box plots: Top: Distribution of the walking duration per run on raw cuticle extract (12 leq). Centre: Box plot of the same values (line within the box represents the median value). Bottom: notched box plots of extract and controls, the indented zone round the median shows the 95% confidence interval of the median.

4. Results

4.1. Publication

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Palmitic Acid Released from Honeybee Worker Larvae Attracts the Parasitic Mite *Varroa jacobsoni* on a Servosphere

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Varroa jacobsoni Oud (Acari; Varroidae), an ectoparasitic mite originating in Asia on *Apis cerana* [1], is currently threatening colonies of honeybees, *Apis mellifera* L., worldwide. If infested honeybee colonies are left untreated, they collapse within 2–4 years due to reduced performance of individual worker bees [2], or fall prey to secondary bacterial or virus infections. By now it is clear that the parasite's life cycle is closely adapted to its host's development. Having finished maturation on a nurse bee [3], adult female *Varroa* enter brood cells of 8–9-day-old worker larvae or 9–10-day-old drone larvae to reproduce, i.e., 20–10 and 40–20 h, respectively, before operculation [4]. *Varroa* slips between the larval body and the wall to the bottom of the brood cell where it immerses in the food jelly until capping is completed by the worker bees [4]. The mite climbs onto the bee larva as it consumes the food [5]. A crucial step for *Varroa* is thus to determine the age of the larva in the brood cell. If the larva is too young, the chance of being detected by nursing bees is high, but soon afterwards the cell will be sealed with the wax cap. To select larvae of the correct age the mites apparently use chemical signals: Three fatty acid esters (methyl palmitate, ethyl palmitate, and methyl linolenate) present in increased amounts in larval cuticle 1 day before operculation [6] attract *Varroa* [7]. However, we could not detect these esters in larval volatiles, but report here on the attractivity for *Varroa* of palmitic acid identified in the headspace over 8-day-old worker larvae.

In order to establish the relative attractivity of different components of the bee hive we bioassayed humid air, wax, adult bees, and larvae. Odors were delivered to *Varroa* on a locomotion compensator [8], and the walking response recorded as follows: The mite walks on the apex of a sphere (50 cm diameter) with a retro-reflective foil (No. 7610, 3M, Switzerland) glued to its back (foil 10–20% of *Varroa*'s body weight). Changes in the position of the mite are recorded by a light-emitting detector system which sends signals to two motors placed orthogonally on the equator of the sphere to compensate for the movement. In this way the mite will always walk on the north pole of the sphere, the location to which odors are delivered. Rotations of the sphere are registered by two incremental pulse generators at a resolution of 1 pulse/0.1 mm displacement. The x and y coordinates of the mite's position are thus sampled at 100-ms intervals and fed into a computer for track record analysis. As all our experiments were carried out in the dark, the visible portion of the light from the detector system was removed with an infrared filter (cut-off at 780 nm).

A chamber built around the servosphere and a treated airstream (ca. 0.05 m/s and 36 mm diameter) directed at its north pole maintained test conditions at 32 °C and 70% r.h. Into these climatized conditions a glass capillary (0.75 mm i.d.) focused an airstream (0.2 m/s) on the place where the mite walked. The capillary bore, alternatively, one of two solenoïd-activated charcoal-filtered airflows: one passing through an empty 50-ml conical glass flask with charcoal-filtered air alone (control), the other passing through a similar flask containing the odor source. Between test runs we allowed headspace volatiles to build up in the flask for 5 min. Silicone tubing, which connected to the glass capillary, and all glassware were replaced between tests with different odor sources. The sphere was rotated between each run to prevent *Varroa* walking on an area already exposed to a stimulus, and the whole sphere was washed intermittently. Walking responses to the different test stimuli were recorded for 10 s following immediately on a control of the same duration. Bees seen to be carrying mites were collected from the hive and kept for no more than 2–5 days in the laboratory. Mites were removed from these bees immediately before testing. Almost all such mites walked at random in the control airstream on the sphere,

with only some showing a slight tendency to walk downstream. When a mite walked down- or crosswind, we switched to the stimulus airstream. The overall direction or vector angle, i.e., the angle between the wind direction (0°) and the straight line joining the start and finish points of a track, was calculated for each 10-s record. A positive response was defined as an upwind-turning mite whose vector angle shifted from a value greater than 60° on either side of due upwind during control to an angle smaller than 30° during test. The small proportion of mites (10–20%) which walked upwind during the control period was eliminated. Responses to the different stimuli were compared pairwise (Fisher-Exact test). Larval odor proved to be the most attractive hive component to *Varroa* (Table 1), but bees also proved attractive.

We then collected volatiles by cold-trapping from larvae that were within 24 h of capping. Air filtered through activated charcoal was passed through a 50-ml conical glass flask containing 50 freshly harvested worker bee larvae at 32°C and was sucked (100 ml/min) through a glass U-tube (5 mm i.d.) that was immersed to 15 cm in acetone/dry ice (−70°C) in a Dewar flask. A 2-h condensate was washed in the U-tube twice with 1.5 ml dichloromethane (Merck, analytical grade) and the recovered solution concentrated by evaporation at room temperature to 1 ml. The response of *Varroa* to this condensate of larval volatiles was tested by applying 100 µl of the extract to a filter-paper disk (5 cm diameter). After evaporation of the solvent, the filter paper was placed in a conical flask above a 20-mm bed of glass beads soaked to 15 mm in water. The latter was introduced to humidify the air to the same extent (60–70% r.h.) as that from larvae. A similar flask plus 100 µl of the solvent alone applied to the filter paper served as control. The mites showed a similar reaction to 100 µl of headspace extracts as to larval odor (Table 1), but not to the solvent control.

The cold-trapped larval volatiles were analyzed by gas chromatography-linked mass spectrometry (GC-MS) with an HP 5890 Series II chromatograph coupled with an HP 5917A mass-selective detector (Hewlett-Packard). A high-resolution HP-1 capillary column (Hewlett-Packard, cross-linked methyl silicone gum, 12 m, 0.2 mm i.d., 0.33 µm film thickness), with helium as carrier gas and splitless injection at 280°C was temperature-programmed after 2 min at 40°C at 20°C/min to 320°C. Detector temperature was 190°C operating in EI mode, and identification was based on comparison of spectral data and retention times of authentic compounds. GC-MS analysis of three cold-trap extracts indicated squalene as well as saturated and unsaturated C_{25}- and C_{27}-hydrocarbons as major components. Palmitic acid (PA) was also detected in each of the three replicates at a maximum of 0.6 ng/larva. None of these products was found in control extracts. Esters of fatty acids reported from the cuticle of 8-day-old larvae in amounts of 55 ng/individual or higher [6] were not found (detection threshold 5 ng on the liquid phase employed). This is surprising in view of the quantities previously detected and the volatility of the esters relative to the acid. However, the esters were reported from washes of the cuticle which may have served to liberate the products more readily than collection by cold-trapping as applied here.

We recorded responses of *Varroa* to dilutions of the major component of the headspace extracts, squalene, as well as to PA, the probable fatty acid precursor of the previously reported attractant, methyl palmitate (MP) [7]. The latter was also included for comparison. Synthetic compouds were dissolved in dichloromethane and applied to filter paper; air in test and control flasks was humidified as described above. A 2.5-µg source of PA was as attractive as the odor from larvae (Table 1). A similar dose of MP evoked a weaker response of *Varroa*, although the difference was not significant (p = 0.12). Squalene proved unattractive. These experiments show that *Varroa* is capable of perceiving volatiles of its host and a component thereof, PA. Most responding mites turned upwind within 5 s of stimulus onset.

In order to study the attraction of *Varroa* to PA in more detail, tracks of 10 mites were recorded for 60 s in a humidified airstream alone and in combination with PA (2.5-µg source). Since displacements constituting individual 100-ms vectors (highest resolution of the servosphere) were too small to ac-

Table 1. Responses of *Varroa* to air (70% r.h.), to the odor of hive components (40 empty brood cells including cocoons of previous molts, i.e., wax, 20 adult worker bees taken from combs with 8-day-old worker brood, 20 8-day-old worker larvae), larval headspace extract (see text), and to squalene, palmitic acid, and methyl palmitate

Odor source	*Varroa* walking cross- or downwind during control	Percentage of upwind turning mites during test[a]
Humid air	34	35.3 cd
Wax	18	38.9 cd
20 live bees	28	67.9 ab
20 live larvae	31	87.1 ab
Solvent	19	26.3 cd
Extract larvae	20	85.0 ab
Squalene [µg]		
25	20	15.0 cd
250	17	41.2 cd
Palmitic acid [µg]		
0.025	18	44.4 cd
0.25	22	68.2 ab
2.5	17	88.2 ab
25.0	31	67.7 ab
Methyl palmitate [µg]		
0.025	37	45.9 cd
0.25	15	40.0 cd
2.5	16	62.5 b
25.0	16	62.5 b

[a] Letters following each treatment indicate significant difference (Fisher-Exact test, $p < 0.05$) from a = humid air, b = solvent, c = larvae, d = extract.

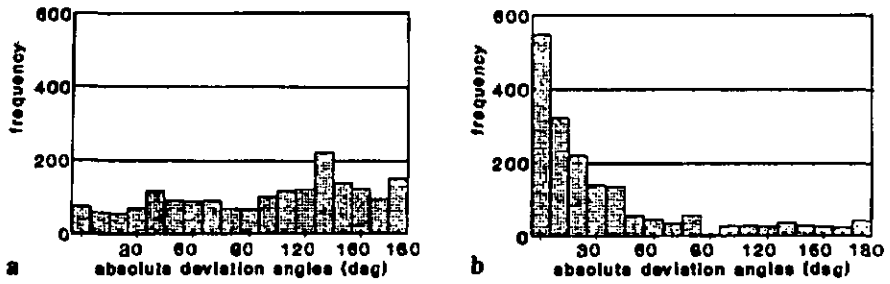

Fig. 1. Frequency distributions of 300-ms vector deviations from due upwind (0°) of 10 *Varroa* responding over 60 s to humidified air (a) and to the same bearing palmitic acid (b). For further explanation, see text

curately reflect the angles described, track sampling was decreased to 300-ms intervals. Vector length, deviation angle from wind direction, and the turn angle (difference in walking direction between successive segments of the track) were calculated for each 0.3-s segment. The frequency distributions of these track parameters in humidified air alone were compared with those in the presence of PA. Means and standard deviations of angular values were calculated as proposed in [9].

The most striking difference occurs in the deviation angles: The mean shifts from 102° (± 49) in humidified air to 40° (± 47) in the presence of PA. When *Varroa* is stimulated with PA, more than 50% of the deviation angles are located between 0 and 30°, whereas in humidified air alone the mites show a more even distribution of deviation angles with even a slight tendency to angles greater than 90° (distributions compared by Kolmogorov-Smirnov test, $p < 0.001$; Fig. 1). The results prove that *Varroa* is capable of closely following an airstream with the stimulus (Fig. 2). This is at least true for a limited period of time, for after some 35 s of upwind walking in the presence of PA we recorded a conspicuous increase in deviation angles with some individuals. Two explanations can be offered: *Varroa* either loses "interest" due to adaptation after some time to the unchanging stimulus conditions, or the concentration of PA in the headspace of our delivery flask dropped too quickly. Turn angles shift significantly from a mean of 23° (± 23) in humidified air to 18° (± 18) during stimulation with 2.5 µg PA (Mann-Whitney, $p < 0.001$). This arises from the fact that the animals walk straighter in the presence of PA. Indeed, path straightness (expressed as the length of the straight line between start and end of a track divided by the total length walked) increased from 0.36 (± 0.14) in humidified air to 0.70 (± 0.20) with PA (Mann-Whitney, $p < 0.01$). Simultaneous video-recording shows that *Varroa* move upwind with a typical zigzag behavior in the presence of PA, i.e., regular shifts of the body axis to the left and the right of the track. This behavior might be responsible for the lower speed of *Varroa* in the presence of PA (2.05 mm/s, ± 1.23) than in humidified air (2.50 mm/s, ± 1.42; Mann-Whitney, $p < 0.001$).

Palmitic acid has been identified in adult honeybee cuticle [10] and, in addition, is suggested to be of use for chemical camouflage by the bee-colony-invading moth *Acherontia atropos* [11]. PA functions as a phagostimulant for the hide beetle, *Dermestes maculatus*, as well as for other insects ([12] and references therein).

According to one hypothesis [13] concerning optimal search strategies in the absence of odors, crosswind walking is expected under constant wind direction, and upwind or downwind walking under variable wind directions. In the absence of an air current, *Varroa* walks randomly on the sphere, whereas the mite shows a slight downwind tendency in a constant airstream of 0.2 m/s (Fig. 1 a). This may be explained by the fact that *Varroa* searching in a bee colony for 8-day-old larvae is permanently surrounded by several potential wind sources, i.e., ventilating bees. Therefore, no other strategy might be necessary than walking randomly until larval odor is perceived and then moving toward the source.

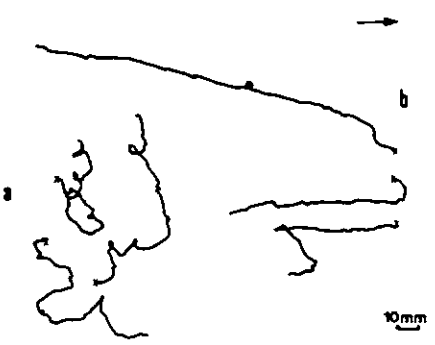

Fig. 2. Track records (60 s) of three *Varroa* a) in a humidified airstream, b) the same bearing PA (2.5-µg source). *Arrow*: direction of airflow; *x*: starting point of path

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Cuticle alkanes of honeybee larvae mediate arrestment of the bee parasite *Varroa jacobsoni* Oud. (Acari; Varroidae)

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ABSTRACT

The ectoparasitic mite *Varroa jacobsoni* invades worker brood cells of the honeybee *Apis mellifera* during the last 20 h 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 uneven-numbered C_{19} to C_{33} 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 behaviour 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.

Keywords: *Varroa jacobsoni, Apis mellifera*, chemoreception, host selection, cuticle, hydrocarbons, alkanes

INTRODUCTION

The ectoparasitic mite *Varroa jacobsoni* (Oud.) threatens colonies of honey bees *Apis mellifera* L. worldwide. It enters brood cells of male honey bee larvae 20-40 hrs and cells of female worker larvae 0-20 hrs before operculation (Boot et al., 1992). Worker bees seal the cells of 9 day-old worker and of 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 timespan of 20 h 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 behaviour appear plausible: a) to avoid being crushed between the larval body and cell wall during cocoon spinning and, b) 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 (cf. Fig.1). In this study, we identify chemostimuli mediating this arrestment response of *Varroa*. Further, 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.

MATERIALS AND METHODS

Use of abbreviations: straight-chain alkanes are abbreviated as *n*-Cx where x indicates the number of carbon (C) atoms followed by the number of double bonds. *Br*-Cx stands for internally branched alkanes, most of them monomethyl alkanes, but also including some dimethyl alkanes. Thus *br*-C25:0 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.

1. Mites

Lots of 60 to 100 *Verroa* visible on the surface of adult bees in heavily infested bee colonies were collected and held for 2-7 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.

2. *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 to 1.0 ml of extract (20 to 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 N2 to a volume equivalent to that of the extract and then tested at a density 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 HC's 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 non-migrating 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 h, 1 g was added to the sample of saturated compounds dissolved in 2-3 ml iso-octane and refluxed at 110-115°C for 8 h. The nonadsorbed *br*-alkanes were recovered from the iso-octane by concentration under N2. Recovery is never complete and in this case some *br*-alkanes were also adsorbed. The initial amount of HC's exposed to the molecular sieve was 50 - 70 leq, the actual amount bioassayed 16 leq *br*-alkanes (12 µg cm^{-2})

3. 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 µm film thickness) was temperature programmed either from 60°C after 2 min (He with 16 kPa headpressure, constant flow) or from 100°C after 2 min (He at 28 kPa, constant flow) at 10° min^{-1} 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 accurately estimate 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 capillary DB-5 column (J&W, California; 0.32 mm id and, 1 µm 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^{-1} to 340°C and held for 16 min or for more detailed analysis from 60°C after 2 min at 5° min^{-1} to 300°C held for 80 min with He at a flow rate of 1.35 ml min^{-1}. 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.

4. Bioassay and data analysis

Three concentric circles of 12, 24 and 36 mm diameter were drawn with a fine 0.1 mm ink pen on the underside of a semi-permeable biological membrane (Baudruche, John Long INC, New Jersey; Fig. 1). This membrane was then washed twice in hexane and once in acetone and streched over a small water bath providing a humidity of ≥ 90 % and a temperature of 32°C on the surface of the membrane. Some 50-200 µl of the test solution were spread with a 10 µl micropipette 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 s or upon leaving the outer circle, which ever came first, the test was terminated and the mite removed.

Synthetic saturated and monounsaturated uneven-numbered *n*-C_{19} to *n*-C_{29} (≥ 97% purity by GC, Sigma) were dissolved in hexane and tested at doses of 30 µg 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 µg 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 µg cm^{-2} of treated area, corresponding to 2-24 leq *n*-alkanes. To test for any

purely physical effect of the HC's on the arena surface, a comparison was made with Vaseline® at 15 µg 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 µg 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 programm linking the video observations with time ("The Observer", Noldus Information Technology, The Netherlands). 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 s on the test band (indexing for a general activity of the animal), 2) showed less than 2.34 returns 10 s^{-1} when moving on the test band (by contrast with chemostimulated mites who 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 the 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.

5. 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*-C21:0 at 30 µg cm^{-2} (Fig. 1) and on the corresponding solvent control (n=10 mites per treatment) were also analysed. Tests chosen for examination of *Varroa*'s tracks are representative of the mite's behavior to chemostimuli 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 5th frame, i.e., every 0.2 seconds 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, or 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 mm. 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/π; 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(Δy/Δx)•180/π (angles for Δx or Δy=0 defined as above) the direction moved per 0.2 s interval (vector) was obtained. The mite's turn angle was calculated as the difference in displacement direction between two subsequent 0.2 s vectors. However, the distribution of turn angles showed a bias for 90° and subdivisions thereof, which is an indication that the distance travelled by the mites (mean speed on solvent control for fraction F1A of 2.85 mm s^{-1}) in the sampling interval chosen was inadequate to properly characterize the true angles described. But 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 s), we still decided to sample at 0.2 s 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 was 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 µg *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 mm s^{-1}) and angular velocity, i.e., the summed absolute turn angles per s. 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 was tested to eight visible fractions including the non-migrating 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 HC's 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 HC's (fraction F1A), the latter was also as active as the total cuticle extract or F1. *Varroa*'s response to an amount of 0.6 leq of the fraction F1A was significantly different than that shown to the solvent control (21% versus 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 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 HC's 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} to 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} to 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 which contained alkanes only, while the other constituents of the extract did not migrate. The branched HC's were harvested from this saturated HC fraction by using a molecular sieve which took up the *n*-alkanes to almost 100 %.

Estimation by GC-FID of quantities of different types of HC's indicated that cuticle extract contains 2.67 µg hexane-soluble material cm^{-2} (sd ± 0.40 µg) of larva. The extract was made up of 75% saturated and 25 % of unsaturated HC's (Fig. 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 ≤1% of cuticle extract or ≤ 0.03 µg per 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 uneven-numbered $C_{19:0}$ to $C_{29:0}$ synthetic *n*-alkanes (Table 2) of neighbouring 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 µg cm^{-2} (50% reacting mites vs 15 % on the control; Fisher-Exact, $p < 0.05$) or higher (Fig. 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 µg cm^{-2} which corresponds to 4.7 leq *n*-alkanes (25% reacting mites vs 7% on the control; $p < 0.05$, $n \geq 20$) or at higher amounts (27% and 41% reacting mites on 15 and 30 µg 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 s vector (no-move vectors 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, $p<0.05$). 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 from 2.85 ± 1.1 mm s^{-1} on controls to 4.06 ± 0.9 mm s^{-1} on 5.9 leq of fraction F1A and decreased their angular velocity from 201.7 ± 82.3° s^{-1} on controls to 179.2 ± 61.0° s^{-1} 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 *n*-$C_{21:0}$ 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. Out of 98 arbitrarily chosen responses on the active cuticle extract, only two responses were designated positive solely for having moved longer than 41.0 s 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 s 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 which 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 in 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 HC's) or higher being behaviorally active. HC fractions of the extract contain, for the most part, uneven-numbered branched and straight-chain saturated and unsaturated HC's; removing the unsaturated compounds did not change the fraction's activity.

Synthetic compounds were only active when saturated HC's were present. Heneicosane at 30 µg cm^{-2} elicited a response similar to the cuticle extract (Fig. 1), but the behavioral threshold for this compound was at 6 µg cm^{-2} or some 200 leq. Since TLC fraction F1A of cuticle extract showed activity at a level some 10 times lower (0.6 leq containing 0.02 µg *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 HC's it contains rather than to a single component. Heneicosane was active at much lower levels (0.04 µg) when presented in a mixture with uneven-numbered C_{23} to 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 HC's 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 are not (Table 2).

The lowest active dose of a mixture of synthetic *n*-alkanes imitating the proportions found in TLC fraction F1A was 6 µg cm^{-2}, but a dose of 0.6 leq of TLC fraction F1A containing 0.76 µg *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 onto 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 HC's 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 homogenously 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 µg 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 which serves to confirm the importance of straight-chain uneven-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 affects *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 to what extent only compounds of purely cuticular origin were extracted. In other studies using the term "cuticular HC's" 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 HC's 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 ≥ 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 avoid 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} HC's are employed to attract females to the vicinity of males (Sato et al., 1993). It is significant, that *Acarapis woodii*, a mite which invades the tracheae of adult honeybees, shows an arrestment response on cuticular HC's 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 ref. therein). Bumble bees, *Bombus terrestris*, mark visited flowers with a secretion from the tarsal glands containing C_{19} to C_{31} HC's; 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 uneven-numbered $C_{21:0}$ to $C_{29:0}$ from host egg masses stimulates oviposition (Grenier et al., 1993).

Saturated and unsaturated HC's 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. 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 which 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 HC's, 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 bees wax (Tulloch, 1980; our own unpublished results). Indeed, extracts of bees wax and adult bees cause arrestment of *Varroa* on the semi-permeable membrane employed here (unpublished results). We must therefore conclude that *Varroa* can recognize bee larvae using HC's only if the relative proportions of saturated HC's employed are sufficiently specific to this life-stage, or in combination with other cues (chemical or otherwise) peculiar to the life-stage 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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Table 1: Responses of *Varroa* to worker honeybee larva cuticle extract and fractions thereof [1)]

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

[1)] For further explanation see text. TLC: Thin layer chromatography separation of extract into front running fraction (F1) and eight other bands (F2 to 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 HC's from branched ones in the saturated HC fraction of the cuticle extract obtained by argentation TLC. Six larva equivalents (leq) of the satura-ted HC's weigh 12 µg whereas 16 leq of the branched alkanes are required to contain as much.

[2] see bioassay and data analysis section of materials and methods.

[3] Within test groups, percentages followed by different letters are significantly different at $p<0.05$ (Fisher-Exact).

[4] highest values obtained per TLC fraction F2 to F9.

Table 2: Response of *Varroa* to synthetic uneven-numbered saturated and unsaturated hydrocarbons (C_{19} to C_{29}) on a circular area [1)]

Compounds	Amount applied per cm^2	No. mites tested	No. runs over at least one limit	% Mites reacting [2)]
(single *n*-alkanes)				
Control	-	34	7	21 a
$C_{19:0}$	30 µg	23	13	57 b
$C_{21:0}$	30 µg	21	15	71 bc
$C_{23:0}$	30 µg	23	5	22 a
$C_{25:0}$	30 µg	30	10	33 ab
$C_{27:0}$	30 µg	50	11	22 a
$C_{29:0}$	30 µg	20	2	10 a
(Binary mixtures)				
Control	-	38	4	11 a
$C_{19:0}$ and $C_{21:0}$	15 µg each	22	13	59 bc
$C_{21:0}$ and $C_{23:0}$	15 µg each	21	16	76 bc
$C_{23:0}$ and $C_{25:0}$	15 µg each	21	17	81 bc
$C_{25:0}$ and $C_{27:0}$	15 µg each	41	30	76 bc
$C_{27:0}$ and $C_{29:0}$	15 µg each	21	7	33 b
(Ternary mixtures)				
Control	-	41	2	5 a
Alkanes $C_{19:0}$-$C_{23:0}$	10 µg each	45	30	67 b
Alkenes $C_{19:1}$-$C_{23:1}$	10 µg each	45	6	13 a

[1)] For explanation see materials and methods.

[2)] within test groups, percentages followed by different letters are significantly different at $p<0.05$ (Fisher-Exact).

Table 3: Track analysis of paths made by *Varroa* on an arena treated either with a nonpolar TLC-fraction of honeybee worker larva cuticle extract (F1A) and one of its constituents, *n*-C21:0.[1)]

Stimulus	Amount applied per cm^2	0.2 s vectors (mean ± sd)		Path segments from one border contact to the next (mean ± sd)	
		Vector length (mm) [2)]	Log of turn angle [2), 3)]	Speed ($mm\ s^{-1}$) [2)]	Angular velocity ($deg\ s^{-1}$) [2), 4)]
Control	-	0.53 ± 0.20 a	3.23 ± 0.87 a	2.85 ± 1.11 a	201.7 ± 82.3 ab
F1A	0.6 leq	0.60 ± 0.28 a	3.25 ± 0.89 ab	3.15 ± 1.14 ab	231.1 ± 54.0 ab
	1.2 leq	0.67 ± 0.32 b	3.16 ± 0.95 ab	3.66 ± 1.16 b	227.4 ± 62.3 a
	2.9 leq	0.70 ± 0.31 b	3.01 ± 0.96 b	3.65 ± 1.03 b	193.8 ± 63.3 ab
	5.9 leq	0.81 ± 0.33 c	2.95 ± 1.02 b	4.06 ± 0.90 b	179.2 ± 61.0 b
Control	-	0.87 ± 0.38 a	3.10 ± 1.19 a	4.81 ± 1.3 a	272.5 ± 58.1 a
n-C21:0	30 µg	1.05 ± 0.41 b	2.83 ± 1.25 b	5.58 ± 1.1 a	205.2 ± 68.2 b

[1)] The differences, especially in the walking speeds, between the solvent controls for F1A and for *n*-C21:0 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.

[2)] 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 dif-ferences attributed serve above all to underline trends with increasing dose.

[3)] Turn angles were calculated from running means generated over triads of consecutive 0.2 s vectors.

[4)] 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.

Figure captations:

Figure 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 µg *n*-C21:0 cm^{-2} (test). Mites were released in the center and their paths drawn for the first 60 s 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 s. Once on the area treated with *n*-C21:0 (b) the mite moved for 147 s on it, returning 61 times to the stimulus after contacting the border. Bar represents 10 mm.

Figure 1

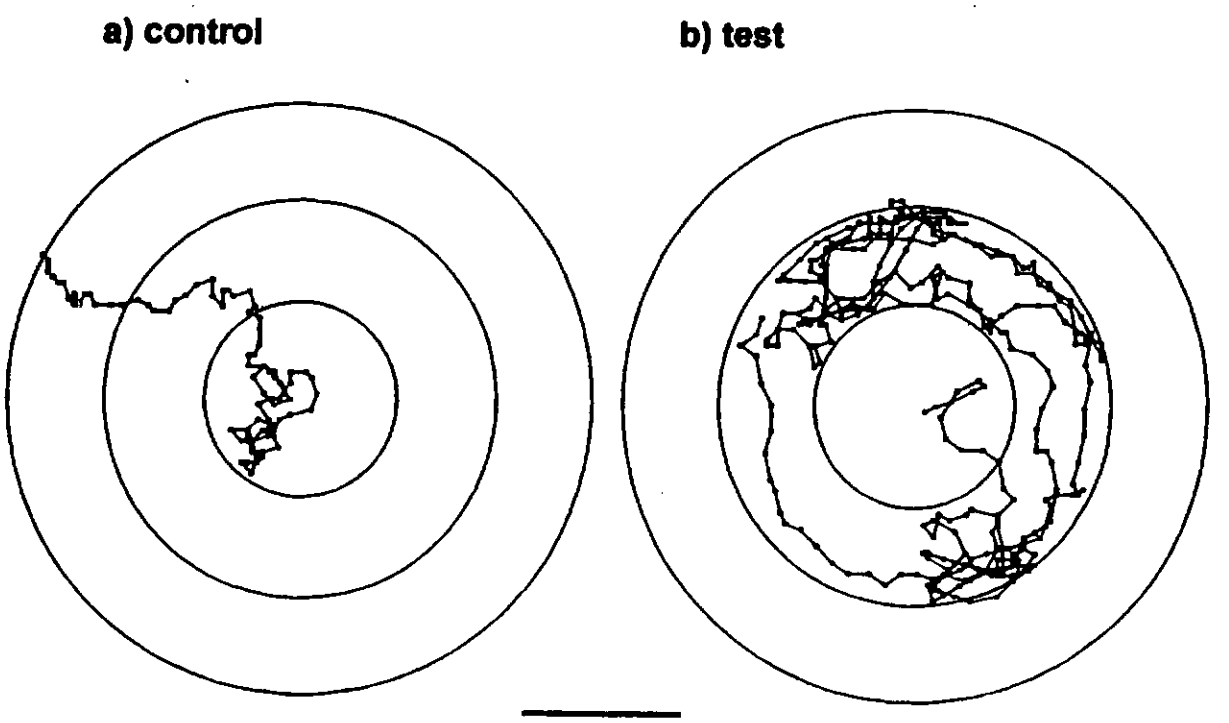
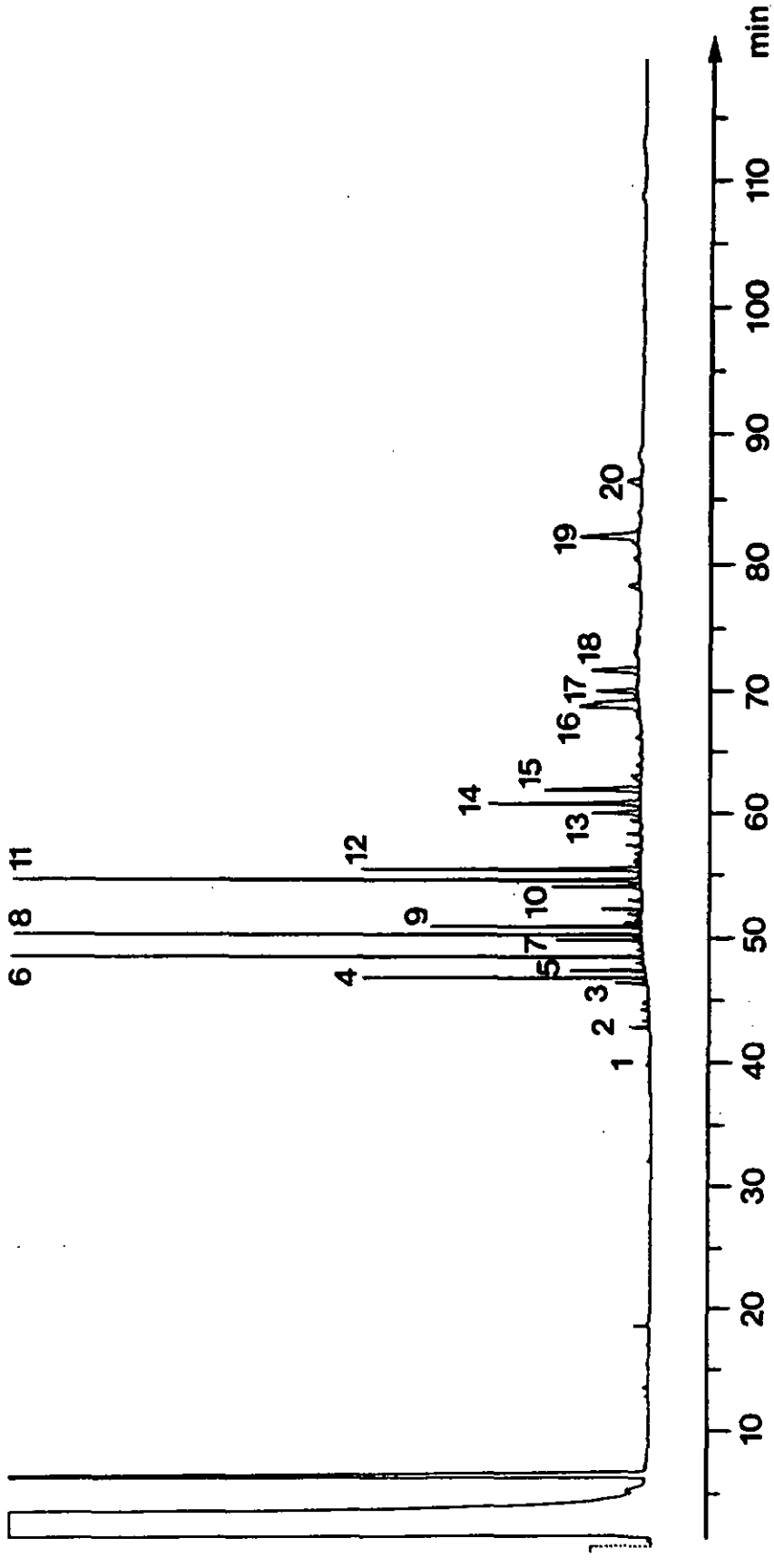


Figure 2: Chromatogram (GC-FID) of cuticle extract of 8 day-old worker larvae on a non-polar DB-5 high resolution capillary column (see materials and methods). The numbered peaks (identified by GC-MS and retention time) show the following compounds: (1) *n*-$C_{19:0}$; (2) *n*-$C_{21:0}$; (3) $C_{23:1}$; (4) *n*-$C_{23:0}$; (5) *br*-$C_{23:0}$; (6) *n*-$C_{24:0}$ (internal standard); (7) $C_{25:1}$; (8) *n*-$C_{25:0}$; (9) *br*-$C_{25:0}$; (10) $C_{27:1}$; (11) *n*-$C_{27:0}$; (12) *br*-$C_{27:0}$; (13) $C_{29:1}$; (14) *n*-$C_{29:0}$; (15) *br*-$C_{29:0}$; (16) $C_{31:1 or 2}$; (17) *n*-$C_{31:0}$; (18) *br*-$C_{31:0}$; (19) $C_{33:1 or 2}$; (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 (± 0.30) µg saturated and 0.69 (± 0.12) µg unsaturated HC's. *n*-Alkanes were found to make up 1.27 (± 0.24) µg and *br*-alkanes 0.73 (± 0.09) µg 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 ≤ 0.03 µg, 0.11 (± 0.03), 0.35 (± 0.15), 0.55 (± 0.22), 0.21 (± 0.09) and 0.08 (± 0.06) µg cm^{-2} of larva, respectively.

Figure 2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4.3. SUMMARY OF RESULTS IN PUBLICATION AND MANUSCRIPT

4.3.1. Palmitic acid released from honeybee worker larvae attracts the parasitic mite *Varroa jacobsoni* on a servosphere

Odour of live 8 day-old larvae proved to be the most attractive hive component to *Varroa*, and cold-trapped volatiles from these larvae gave a similar response. GC-MS analysis of the cold-trap condensates indicated the presence of squalene as well as C_{25} and C_{27} HC's as major components and palmitic acid (PA) as a minor component. Squalene and the fatty acid were tested and only the latter proved attractive to *Varroa*. A similar dose of methyl palmitate (MP) evoked a weaker response although the difference was not significant (Fisher's exact test; $p=0.12$; described in chapter 4.1).

The walking behavior of *Varroa* stimulated by PA on the servosphere changed to an upwind walk whereas in humidified air alone a slight acrosswind preference was recorded. Statistics on turn angles of 1 min runs under the conditions of humidified air alone and the same bearing PA had been made using the frequency of relative turn angles (left/right shifts of the body axis) in classes of 5° each. These angles shift significantly closer to 0° (straight ahead) in air bearing palmitic acid (Mann-Whitney Test, $p<0.001$). The walking speed was reduced in PA bearing air.

4.3.2. Cuticle alkanes of honeybee larvae mediate arrestment of the bee parasite *Varroa jacobsoni* Oud. (Acari; Varroidae)

Raw cuticle extracts of worker larvae had an arrestment effect on the mite. The mites walk for prolonged periods on the extract and the returns at the borders of areas treated with the extract proved crucial for recognition of the chemostimulus. The most apolar TLC fraction (F1) elicited similar reactions as raw cuticle extract. In fraction F1 alkanes (saturated hydrocarbons, HC's), alkenes (mostly mono-unsaturated HC's) and wax esters ($C_{16:0}$- or $C_{18:1}$-fatty acids esterified with C_{24-32} alcohols) were identified by GC-MS. Removing the wax esters did not change the HC's activity, i.e., the TLC subfraction F1A was active. Alkanes alone, obtained by argentation TLC from the raw extract were active, but unsaturated compounds inactive in our bioassay. *Br*-alkanes harvested from the argentation TLC alkane fraction with a molecular sieve did not elicit the typical reaction at the area borders of the treated area but did prolong the mites' walk on the treated area (described in chapter 4.2.).

Combinations of 2 or 3 synthetic *n*-alkanes of neighbouring chain lengths (uneven numbered, C_{16} to C_{29}) predominating in the active TLC fraction F1 and in subfraction F1A and in the alkane fraction of the argentation TLC were also active, but most of these *n*-alkanes were inactive when tested alone. However, *n*-$C_{21:0}$ alone did elicit a response similar to cuticle extract. The behavior threshold for this compound was ≥ 6 µg cm^{-2} whereas the purified TLC subfraction F1A of cuticle extract showed activity at 0.6 leq containing 0.02 µg *n*-$C_{21:0}$ cm^{-2}.

PA, the *Varroa* attractant (see chapter 4.1), presented alone on the membrane was inactive. PA mixed with alkanes did not significantly alter the HC's activity. TLC fraction F7 which contained free PA in addition to other fatty acids was also inactive. MP also was inactive whereas the TLC fraction F5 containing nonanal, methyl and ethyl fatty acid esters and the corresponding fatty

acids elicited no response in terms of border recognition but longer stopping durations compared with the control (notched box plots, Fig 7).

4.4. Unpublished results

4.4.1. Anemotaxia of *Varroa* in the absence of chemostimuli on the servoaphera

Under constant wind conditions across- or downwind walking mites showed no difference between spontaneous upwind turning rates in air of <0.05 m s^{-1} (13%) or in 0.2 m s^{-1} (9%, Fisher-Exact test; p = 0.22; 10 s path segments). Switching from one to a second airflow both of 0.2 m s^{-1} did not increase the rate of upwind turning mites (15 %, Fisher-Exact p = 0.12) compared to a continuous airflow of the same speed. However, 50 % of across- or downwind oriented mites responded with upwind walking to an increase of the airspeed from < 0.05 to either 0.1 or to 0.2 m s^{-1} (Table 2).

4.4.2. Preliminary experiments on the zoned membrane

When 12 larva equivalents (leq) per cm^2 of the hexane extracts of larvae were applied to filter paper, to glass and to the Baudruche membrane the mites only showed an arrestment response on the membrane as described in chapter 4.2. (n = 20 mites per treatment). Temperature and r.h. were the same in all three cases on the substrate (32°C and ≥ 90%). These trials were not quantified.

In addition, methanol and dichloromethane extracts of 8 day-old larvae (12 leq per cm^2) were either less active or had an activity similar to the hexane extracts. These tests were not quantified.

If - as during a hot summer day - the temperature was allowed to reach 27°C in the laboratory where the mites were held prior to bioassays for up to 7 days and where preliminary bioassays were carried out, then the mites spent some 90% of the time on the membrane immobile. The pro-

Table 2: *Reaction of* Varroa *to changes in airspeed during short (10 s) stimulation*

Airspeed in control (m s^{-1})	Airspeed in test (m s^{-1})	No. *Varroa* walking across- or downwind in control	No. *Varroa* walking upwind in test*
<0.05	0.1	15	8 a
<0.05	0.2	17	9 a
0.2	0.2	20	3 b

* *different letters indicate a significant difference (Fisher-Exact-Test, p<0.05).*

portion of time spent immobile dropped to 20% when the mites were held at room temperatures of 19 to 21°C at which the bioassays subsequently were carried out.

4.4.3. Zoned membrane: Analysis of walks in contact-chemoorientation tests

Three parameters of 671 runs on controls with highly asymmetric value distributions were used to define the standard walking pattern on the test arena (described in 4.2.). If a mite showed for one of the three parameters a value which was observed in ≤ 5% of the control runs it was considered to respond to the material applied to the treated area. Because these criteria are so fundamental to this study the distributions of the three parameters used, i.e., time moving on the treated area, number of returns per 10 s walk on the treated area and number of returns per run are described in Fig. 5.

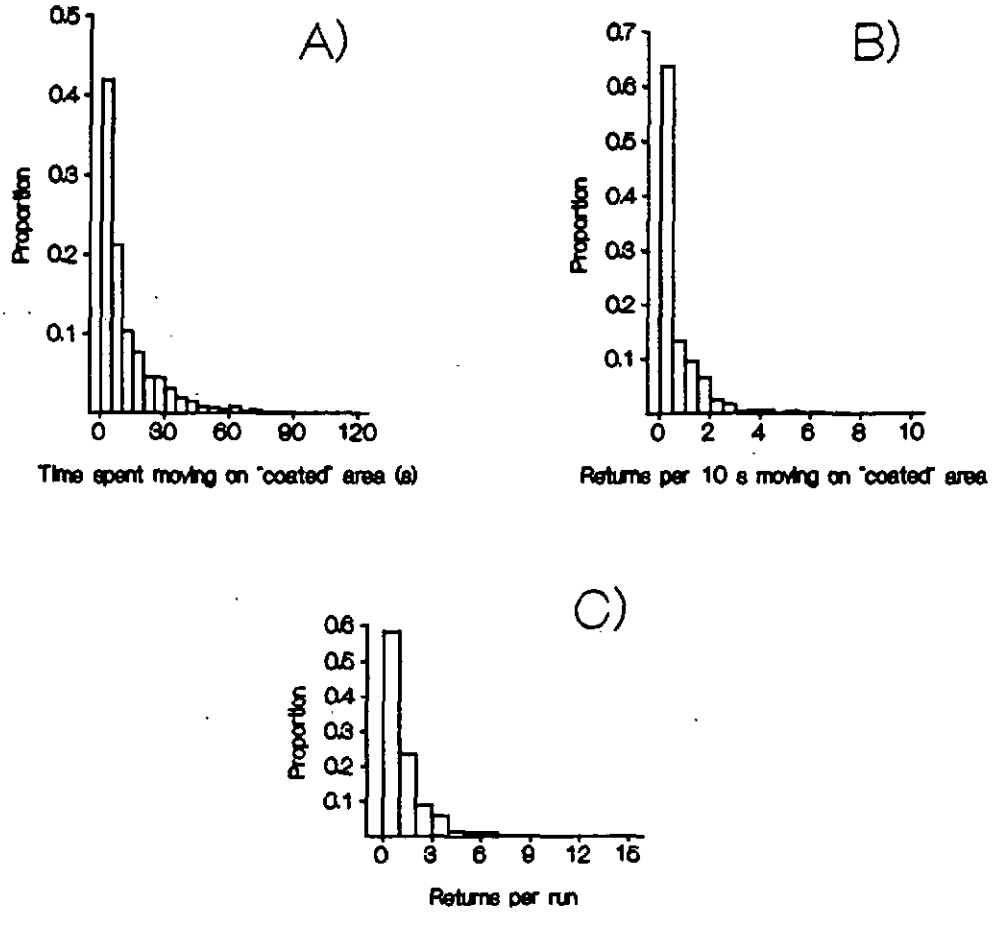

Figure 6: Distributions of the 3 parameters used to define a standard walking behevior on the zoned membrane treated with solvent only (n = 671 runs). A) For time spent moving on the "coated" area 95 % of the mites had values below 41.0 s. B) For returns per 10 s moving on the "coated" area 95 % of the mites had values below 2.34. C) In the case of the number of returns made at the border resulting in continued contact with the "coated" area 95 % of the mites showed less than 4 returns per run.

4.4.4. Zoned membrane: Responses to extracts of adult bees and wax

Chemical analysis of the extracts of 8 day-old larvae, adult bees and comb wax (see chapter 4.4.9.) showed straight-chain alkanes C_{21} to C_{33} to be common to all three extract sources. These extracts were therefore expected to be behaviorally active and this was confirmed in bioassays (Table 3).

4.4.5. Zoned membrane: Responses to attractants

The attractive odour compounds of larvae, MP (LeConte et al., 1989) and PA (Rickli et al., 1992) are inactive as contact-chemostimuli at amounts of 1.5 µg MP and 3 µg PA cm^{-2} (Table 4). Adding PA to a binary mixture of C_{25} and C_{27} *n*-alkanes found in condensates of larval volatiles, did not alter the acitvity of these HC's on the zoned membrane.

Table 3: Responses of Varroa to hexane extracts of adult bees taken from the brood nest and to the wax of empty cells in the brood nest (extraction time for both 15 min)

Extract	Amount applied per cm^2	No. mites tested	No. runs over at least 1 limit	% Mites reacting [1)]
Control	-	20	3	15 a
adult bees	0.29 bee equ.	20	10	50 b
comb wax	0.15 cell equ.	20	18	90 c

1) percentages followed by different letters are significantly different at $p<0.05$ (Fisher-Exact)

4.4.6. Notched box plots

The notched box plots have some relevance for assessment of the activity of two fractions which are not active in terms of border recognition (Table 1 in chapter 4.2). These fractions demonstrated activity in terms of total walking duration on the treated area as for the branched HC's after adsorption of the straight-chain alkanes to the molecular sieve (Fig. 6d) or in terms of stopping duration on the treated area for TLC fraction F5 (Fig 7a). For purposes of comparison notched box plots of the activity associated with the main fractions obtained during the chemical separations are shown as well.

Table 4: Responses of Varroa to methyl palmitate and palmitic acid, the latter admixed with the alkanes n-C25:0 and n-C27:0.

Compounds	Amount applied per cm^2	No. mites tested	No. runs over at least 1 limit	% Mites reacting [1)]
Control	-	20	3	15 a
Methyl palmitate	1.5 µg	20	3	15 a
Control	-	32	3	9 a
Palmitic acid (PA)	3µg	21	0	0 a
PA and *n*-C25:0	3+27µg	22	2	9 a
PA and *n*-C27:0	3+27µg	20	5	25 ab
n-C27:0	30 µg	20	2	10 a
n-C25:0 and *n*-C27:0	each 15 µg	23	13	57 bc
PA, *n*-C25:0 and *n*-C27:0	3+13+13 µg	21	16	76 c

[1)] within test groups, percentages followed by different letters are significantly different at $p<0.05$ (Fisher-Exact)

4.4.7. Zoned membrane: Walking behavior on subfraction F1A of larva extract

a) Border contacts: Although the duration of border contacts decreased at higher stimulus doses, no significant difference between the treatments was noted (Tukey-HSD Test on the logarithmized durations). Some 90 % of all border contacts were shorter than 1.6 s. Neither was the total (2.5 ± sd 1.03 mm on control and 2.2 ±1.1 mm on 5.9 larva equivalents, leq) nor the net displacement (1.3 ± 0.64 mm on control and 1.3 ± 0.78 mm on 5.9 leq) along the border significantly different between treatments.

On doses ≥ 1.2 larva equivalents (leq) the total number of returns shown by 10 mites (per dose) and specifically the number of returns with continuous walking increased dramatically (Fig. 8). These returns have a cumulative character, i.e., if a mite shows a few returns then the probability that it will show some more returns is high due to border recognition. Therefore statistical analysis should not be used on these numbers. The number of 10 mites recorded per dose is too low to allow similar statistics as described in chapter 4.2.. However, the differences between control and 0.6 leq on the one hand and of 1.2 leq or above on the other are obvious and require no statistical analysis (Fig. 8). The majority of all returns observed on doses ≥ 1.2 leq were made while walking.

b) Parameters of the walks made on the treated area and on its borders: The mean length per 0.2 s vector (no-move vectors excluded from calculations) on the treated area increased significantly with stimulus dose (Table 5). This increase was less pronounced on the borders where only 5.9 leq elicited vector lengths significantly longer to controls. Vectors on bor-

ders of 1.2, 2.9 and 5.9 leq were, however, significantly shorter than on the treated area. The mean of turn angles on the treated area decreased significantly from 25° (log values reconverted into degrees) on controls to 19° on 5.9 leq (Table 6). No significant difference between treatments was observed for turn angles described at the borders. Although the mean turn angles at the borders were in general greater than on the substrate, a significant difference was found only on 1.2 leq. In general, the mites walked faster and straighter on higher doses of the stimulus, and on patch borders they slowed down significantly but a dose-dependency in turn angles was not observed here.

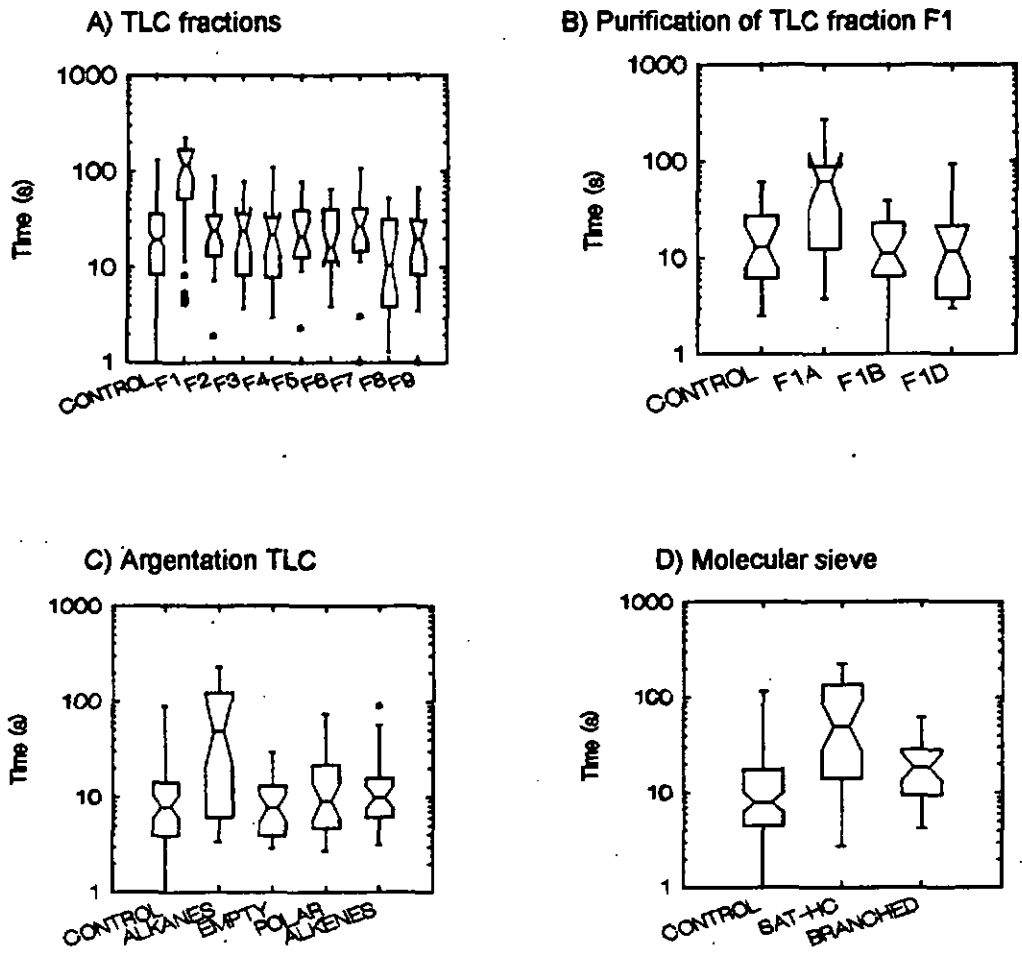

Figure 6: Box plot presentation of the activity associated with different fractions during chemical separation steps in terms of time walking on the treated area (including borders). A) TLC fractions: F1 to F9 of the total extract of 8 day-old worker larvae. B) Purification of TLC fraction F1 by eluting F1 in hexane alone resulting in a front-running subfraction F1A containing the HC's alone, wax esters in subfraction F1B and F1D is the TLC start band. C) Argentation TLC of total cuticle extract: front-running fraction of alkenes, followed by an empty stripe, whereas the more polar compounds stayed at the starting TLC band. Alkenes were purified from that starting TLC band by eluting it alone in hexane on ready made TLC plates. D) Molecular sieve: saturated HC's (SAT-HC) obtained by argentation TLC were exposed to a molecular sieve to which the straight chains adsorbed. The branched chains remained in solution, were recovered and tested (see chapter 4.2.).

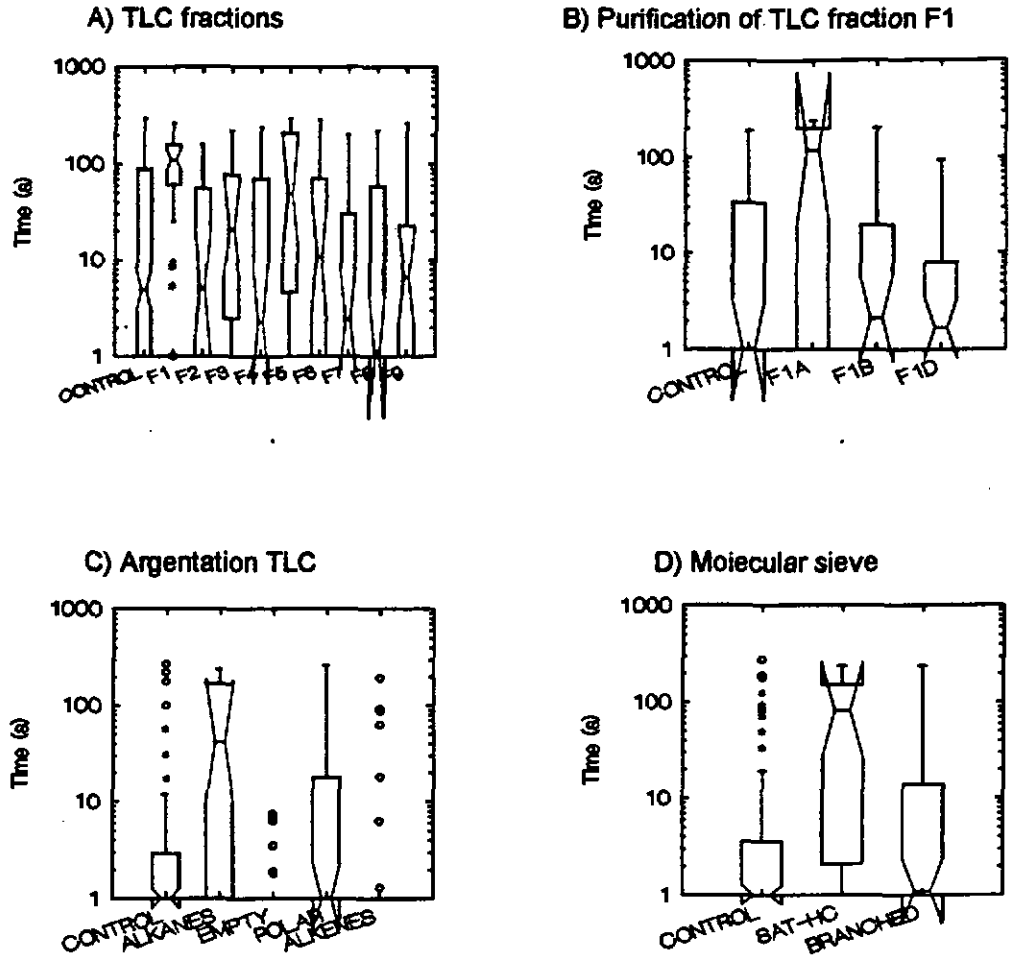

Figure 7: Box plot representation of the activity associated with different fractions during the chemical separation as in Fig. 6, but in terms of time spent immobile *on the treated area (including borders).*

Table 5: Mean length of 0.2 s vectors made by Varroa walking on an area treated with TLC fraction F1A of honeybee larval extract and on its borders.

Dose	Vector length on treated area (mm; mean ± sd) [1]	No. of vectors on treated area [2]	Vector length on border of treated area (mm; mean ± sd) [1]	No. of vectors on border of treated area [2]
Control	0.53 ± 0.20 a	359	0.52 ± 0.22 a	214
0.6 leq	0.60 ± 0.28 a	189	0.57 ± 0.25 ab	128
1.2 leq	0.67 ± 0.32 b^{+}	758	0.60 ± 0.29 ab^{+}	304
2.9 leq	0.70 ± 0.31 b^{+}	795	0.55 ± 0.26 ab^{+}	343
5.9 leq	0.81 ± 0.33 c^{+}	771	0.62 ± 0.27 b^{+}	192

[1] different letters indicate significant differences (vertical comparison; Tukey-HSD, $p<0.05$) and ($^{+}$) significant difference between vector lengths on treated area versus border of the respective dose (horizontal comparison; Tukey-HSD, $p<0.05$).
[2] No-move vectors excluded from the calculation.

4.4.8. Analysis of *Varroa* behavior at the borders of an area treated with a contact chemostimulant

Mites returning at the border on controls or on 0.6 leq showed high angles to the border tangent of -66° and -55°, respectively (the minus sign indicating return from the border back onto the treated area, Table 7). These angles decreased significantly to -45° on 1.2 and 2.9 leq and to -36° on 5.9 leq (Tukey-HSD, $p<0.05$). The angle to the tangent at arriving at the border was also dose-dependent.

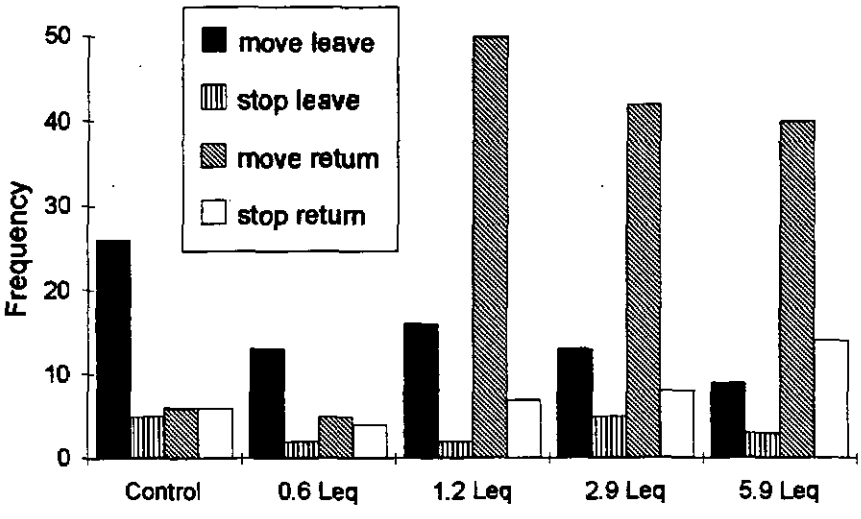

Figure 8: Frequency of border contects with stops (stop) or continuously walking (move), and of returns back to (return) and departures from the treeted area (leeve) on different doses of TLC fraction F1A and control.

Table 6: Turn angles (log values) of 0.2 s vectors made by Varroa *walking on an area treated with TLC fraction F1A of honeybee larval extract and on its borders.*

Dose	Log of turn angles on treated area (mean ± sd) [1]	No. of turn angles on treated area [2]	Log of turn angles on border of treated area (mean ± sd) [1]	No. of turn angles on border of treated area [2]
Control	3.23 ± 0.67 a	245	3.39 ± 0.69 a	147
0.6 leq	3.25 ± 0.89 ab	148	3.42 ± 0.86 a	98
1.2 leq	3.18 ± 0.95 ab[+]	629	3.39 ± 0.69 a[+]	243
2.9 leq	3.01 ± 0.96 b	631	3.17± 0.86 a	262
5.9 leq	2.95 ± 1.02 b	645	3.09 ± 0.96 a	143

+ significant difference between turn angles on treated area versus those made on the border of the same dose (horizontal comparison; Tukey-HSD, $p<0.05$)
[1] different letters indicate significant differences (vertical comparison; Tukey-HSD, $p<0.05$);
[2] Turn angles were calculated as running means over triads of consecutive 0.2 s turn angles. Any triad with a no-move vector was excluded from the calculation.

Table 7: Angles to tangent made by Varroa *on arriving at and departing from the border of a circular area treated with honeybee larval extract and with solvent only (all returns) [1]*

Dose	Angle of arrival vector to border tangent for all border contacts (mean ± sd) [2]	No. arrivals	Angle of departure vector to border tangent for returns only (mean ± sd) [2]	No. returns
Control	57.2 ± 17.9 a	34	-66.0 ± 23.3 a	12
0.6 leq	52.4 ± 24.1 ab	20	-55.0 ± 17.7 ab	7
1.2 leq	47.3 ± 25.0 ab	67	-45.3 ± 24.4 b	50
2.9 leq	47.1 ± 23.2 ab	66	-45.6 ± 22.4 b	50
5.9 leq	41.2 ± 24.1 b	54	-35.6 ± 19.9 b	45

[1] Three successive vectors, each of 0.2 s, were summed giving 0.6 s arrival and 0.6 s departure vectors at the border of the treated area. Arrivals or departures where more than one 0.2 s vector was 0 (no move) were excluded from the calculation, which accounts for differences to frequencies shown in Fig. 8. The minus sign indicates departure from the border back onto the treated area.
[2] different letters indicate significant differences (vertical comparison; Tukey-HSD, $p<0.05$).

I first tested the hypothesis that the underlying mechanism could be a billiard ball effect, i.e., that for returns of ≤ 0.2 s the angle of arrival equals the angle of departure, but detected no significant correlation between arrival and departure angles on 5.9 leq F1A (Fig. 9a).

The sum of arrival plus departure angles to the tangent at the border (absolute values) represents the rotation of the direction of displacement between arriving and returning (correction angle). First, corrections made within 0.2 s on 5.9 leq F1A were analyzed and a significant cor-

relation with the arrival angles was observed (r = 0.874, df = 27, p<0.01, Fig. 9b). For returns made after a longer duration at the border - with a maximal displacement along the border of 3.4 mm - the correlation was lower but still significant (r = 0.768, df = 16, p<0.01). On lower doses of extract i.e. 1.2 and 2.9 leq the correlation between arrival and correction angle was lower than on 5.9 leq for both 0.2 s and for those occurring after longer border contacts, but they were still significant (p<0.01) except for returns after 0.2 s contacts on 1.2 leq.

Plotting the correction angles for inner and outer border contacts separately with the respective arrival angles gave lower regression lines and lower slopes for the inner border contacts on 5.9 leq F1A (y = 20.96 + 1.22 x; r = 0.816, df = 14, p<0.01) than for the outer ones (y = 30.42 + 1.19 x; r = 0.862, df = 27, p<0.001). Further, the means of the correction angles were systematically lower for the inner border contacts of each dose than for the outer border contacts (75.6° versus 104.3° on 1.2 leq, 84.0° versus 101.4° on 2.9 leq and 74.7° versus 78.9° on 5.9 leq), but these differences between the correction angles at the inner and outer borders were not significant.

The minimal correction angles which are necessary to re-establish full contact with the stimulant after contacting the border either with P1 only or with the palps in a simplified model are given in Table 6. For arrival angles between 30 and 60° the correction angles required to re-establish full contact after touching the border with one P1 alone were smaller that the arrival angles. This means that in these cases the "model-mite" rotated its body axis a certain angle to come fully on the stimulating surface again. Nevertheless, after such a correction its body axis was still directed *toward* the border and the animal still moved *toward* the border in our model. Thus, the minus-sign indicating movement *away* from the border was not assigned to these directions of displacement ("departure angles"). The departure

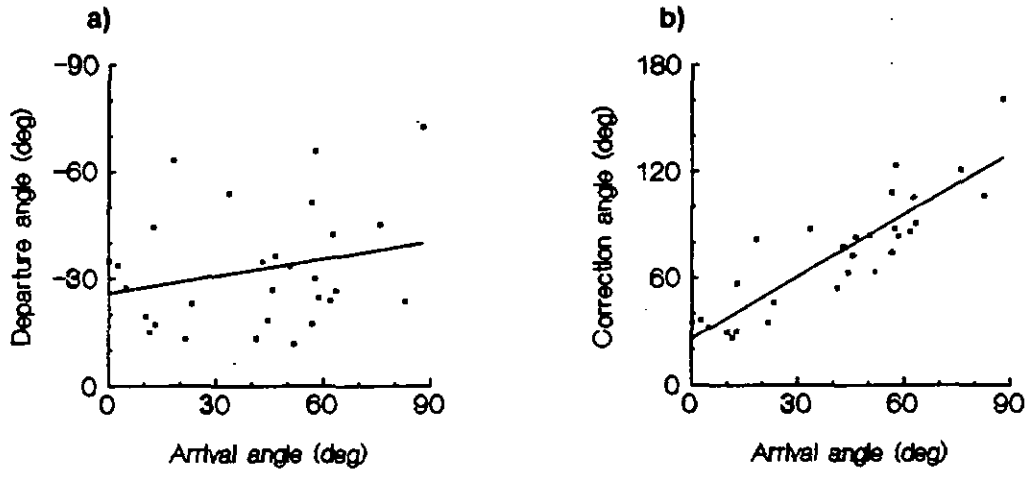

Figure 9: Plots of arrival angle and the return angle to tangent at the point of contact (a) and the size of the correction angle (b) of returns made within 0.2 s by Varroa *at the borders of an area treated with 5.9 leq. Following linear regression the Person's correlation coefficient indicated an r value of -0.241 for a) and 0.874 for b).*

angles measured in the model for returning after contacting the border with one P1 alone were all between +9 (see above) and -20°. Departure angles for returns after contacting the border with the palps were found between -17 and -44°. The observed correction angles for returns within 0.2 s fit into the range delimited by the model for arrival angles of < 60° (Fig. 10). The mites correct their walking direction more than predicted by the model for angles > 60°.

Of all 144 cases of returns made during 5 min test runs on 5.9 leq of TLC subfraction F1A where the mites approached the border at angles of 60° or lower, 85 % of turns following border contact were made towards the extract and only 15 % away from the extract. These numbers are significantly different from an equal distribution (Fisher-Exact, $p < 0.001$). Furthermore, the size of the angles to tangent when arriving at the border before *returns* was not different from arrival angles before *leaving* the treated area (Tukey-HSD, $p > 0.2$)

4.4.9. Analysis and Identification of surface products of host, *Varroa* and wax

Extracts of 8 day-old larvae and of washes of the walls of their brood cells were analyzed by GC-FID (Fig. 11 and 12). A comparison of surface products of larva cuticle and cell walls analyzed here and of freshly emerged and 6 day-old adult bees by Francis et al. (1989; Fig. 12) hinted to the common occurrence of alkenes, *n*- and *br*-alkanes on all substrates. However, some differences in the relative amounts of the compounds have been found. Unsaturated HC's shift from low proportions in larvae and freshly emerged bees to higher proportions on cell walls and on 6 day-old adult bees. Alkanes have longer chains on adult bees, and *br*-alkanes are present in far higher amounts on larvae and freshly emerged bees than on 6 day-old bees or on cell walls. These comparisons are to be understood only as hints, not as evidences since the results were not confirmed by statistical analysis. Quantification of the constituents of raw cuticle extracts of larvae are shown in Table 10.

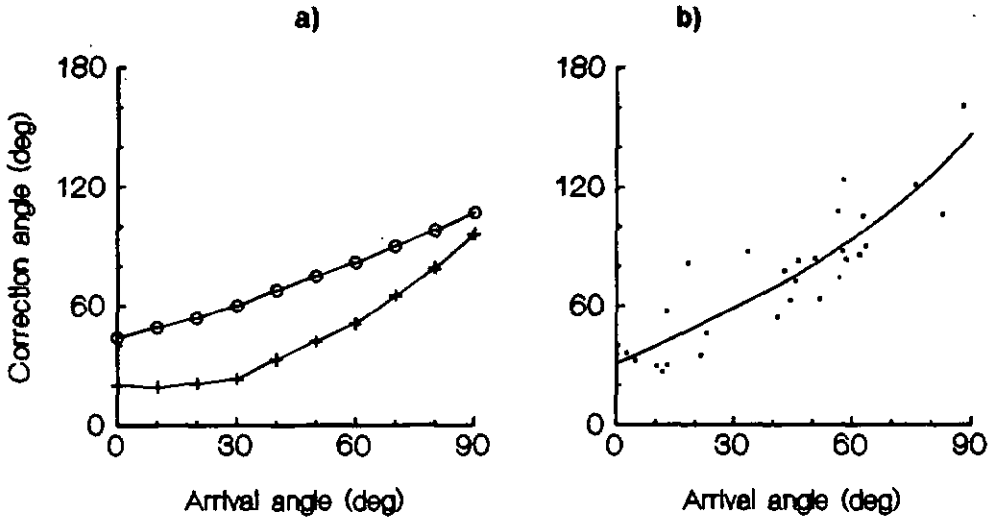

Figure 10: Correction angles a) predicted by a simple model for contacts with a straight border (+ turning after border contact with one P1; o turning after border contact with palps) and b) values observed on a circular area treated with 5.9 leq TLC subfraction F1A (contacts of ≤ 0.2 s; line calculated with "distance weighted least square" method, Systat®).

***Table 8:** Results of a simple model with a dummy mite contacting a straight border at different arrival angles. Departure angles and therefore the size of the corrective action are higher if the mite turns on contacting the border with the palps than after contacts with one P1 only [1)]*

	after contact with P1		after contact with palps	
Arrival angle (deg)	"Departure" angle (deg)	Correction angle (deg)	Departure angle (deg)	Correction angle (deg)
0	-20	20	-44	44
10	-9	19	-39	49
20	-1	21	-34	54
30	7 *	23	-30	60
40	7 *	33	-28	68
50	8 *	42	-25	75
60	9 *	51	-22 §	82
70	5 *	65	-20 §	90
80	1 *	79	-18 §	98
90	-6 §	96	-17 §	107

1) The minus sign signifies return from border back onto the treated area.
§ The two P1 simultaneously contact the border.
** Although the "mite" re-established full contact with the stimulant, its body axis was still directed towards the border and thus no minus-sign was assigned to these "departures" from the border. Consequently the correction angles here are smaller than the arrival angles.*

The cuticle of adult worker bees of different ages was analyzed by GC-MS together with mites removed from them. Mites had more fatty acids (Fig. 13b and d; eluting before peak 4) than bees (Fig. 13a and c), but less wax esters ($C_{18:0}$ or $C_{18:1}$ fatty acids esterified with C_{24-32} alcohols eluting at retention times >23.9 min or after peak 19). On both, freshly emerged workers and their mites higher proportions of *br*-alkanes were present than on bees taken from the brood nest. *Varroa* from brood nest bees have proportions of *br*-alkanes resembling more newly emerged bees than their latest host. A close fit between cuticles of host and parasite was observed for the alkenes $C_{31:1}$ and $C_{33:1 \text{ and } 2}$.

The ontogeny of the cuticular HC's of honeybees might provide hints as to what changes in the chemical environment *Varroa* is exposed to, and what cues might be available to them. Extracts of 8 day-old worker larvae, 6 to 7 day-old pupae, freshly emerged bees and hive bees of mixed age from a brood comb were compared by GC-MS. The immature stages (Fig. 14a and b) had high proportions of *br*-$C_{25:0}$ which decreased in adult bees (Figure 14c and d). *Br*-$C_{27:0}$ *br*-$C_{29:0}$ and *br*-$C_{31:0}$ were present at high amounts except on hive bees of mixed age, whereas *br*-$C_{33:0}$ was present in substantial amounts only on larvae. Further, the chromatograms confirmed data on the ontogeny of adult worker cuticle

(Francis et al., 1989), namely the high proportion of *n*-C23:0 on newly emerged bees. A marked increase of the alkenes C31 and C33 together with an increase in wax esters (eluting after peak 19 or 21 in Fig. 14) was observed in the cuticle extract of adult bees compared to larvae or pupae.

4.4.10. Fertility of the mites used in bioassays

Some 116 mites from hive bees and 177 from newly emerged bees (<24 h) were artificially inserted into brood cells cappped for 12 hrs or less (Rosenkranz, 1990), the brood returned to the hives. At 6 days before emergence of the bee the cells were opened and checked for reproductive success of the mites (Fuchs and Langenbach, 1969). 52% of the cells with mites from the hive bees contained deutonymphs which could have terminated their development till the bee's emergence, whereas when newly emerged mites were inserted only 35 % had offspring (Fischer-Exact-Test; $p<0.005$).

Table 9: *Compounds found in extracts of larvae, adult bees and of wax (identified by GC-MS in extracts of larvae and adult bees and matching GC-FID retention times). Br-Cx:0 stands for internally branched alkanes, most of them mono- but also some dimethyl alkanes. Alkanes are straight chain and mono-unsaturated except for small quantities of C31:2 and C33:2.*

Compound	Retention time (min) 1)	Identification number in chromatograms	Compound	Retention time (min) 1)	Identification number in chromatograms
n-C19:0	39.93	1	*br*-C27:0	55.47	12
n-C21:0	42.96	2	C29:1	60.07	13
C23:1	46.44	3	*n*-C29:0	60.84	14
n-C23:0	47.84	4	*br*-C29:0	61.98	15
br-C23:0	47.42	5	C31:1	68.78	16
n-C24:0 *	46.84	6	*n*-C31:0	70.03	17
C25:1	49.84	7	*br*-C31:0	71.70	16
n-C25:0	50.27	6	C33:1	82.07	19
br-C25:0	50.91	9	*n*-C33:0	-	20
C27:1	54.09	10	*br*-C33:0	86.07	21
n-C27:0	54.65	11			

1) Retention times in GC-FID. GC-conditions as described in chapter 4.2. (detailed analysis)
** internal standard*

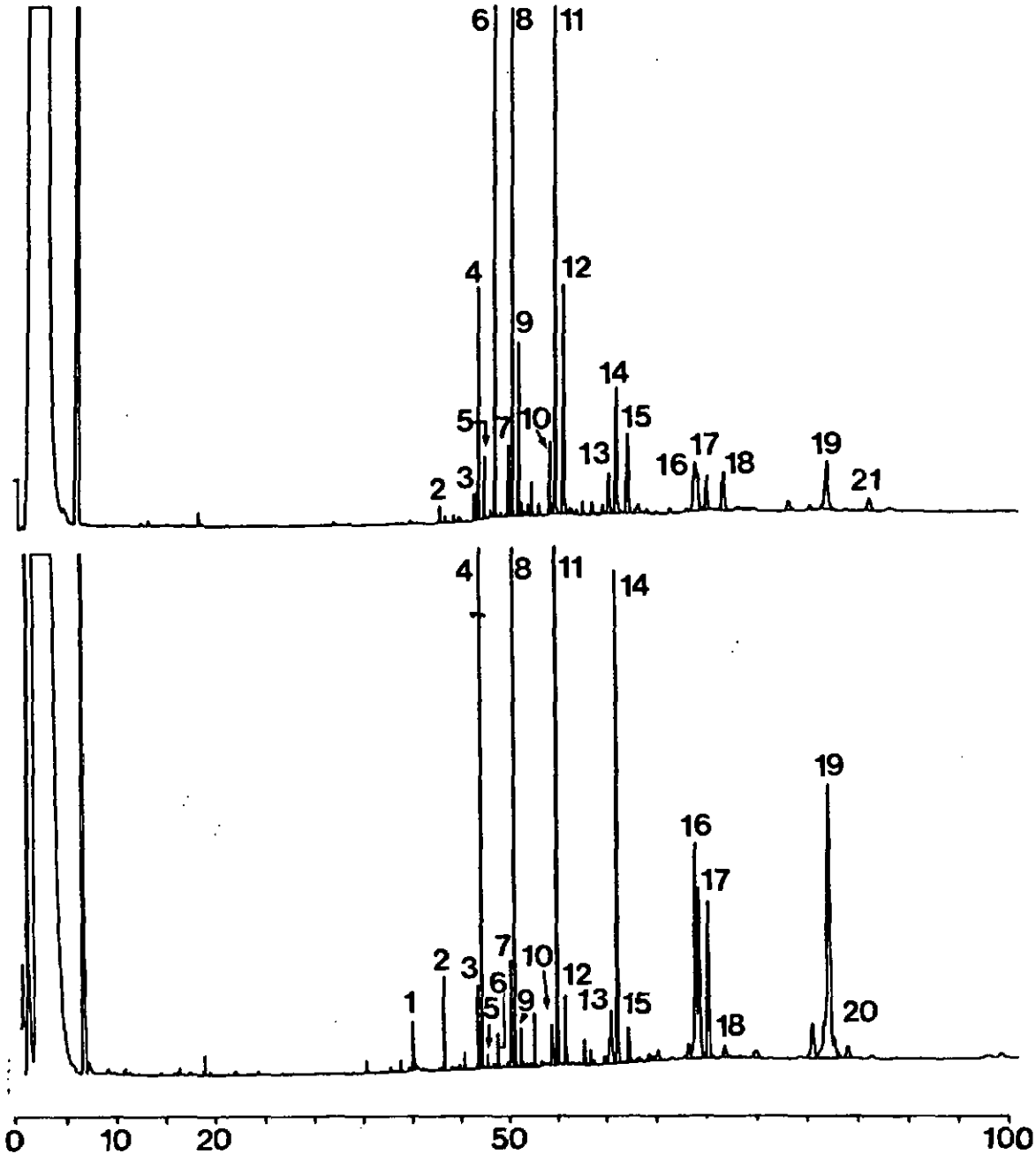

Figure 11: Chromatograms (GC-FID) of raw cuticle extract of 8 day-old worker larvae (top) and of wax of the cell walls occupied by such larvae (bottom). The walls of brood cells had been rinsed 3 times with 0.5 ml hexane after removal of the larva and the extract concentrated before injection. Identification of numbered peaks as in Table 9 (x-axis = retention time in min).

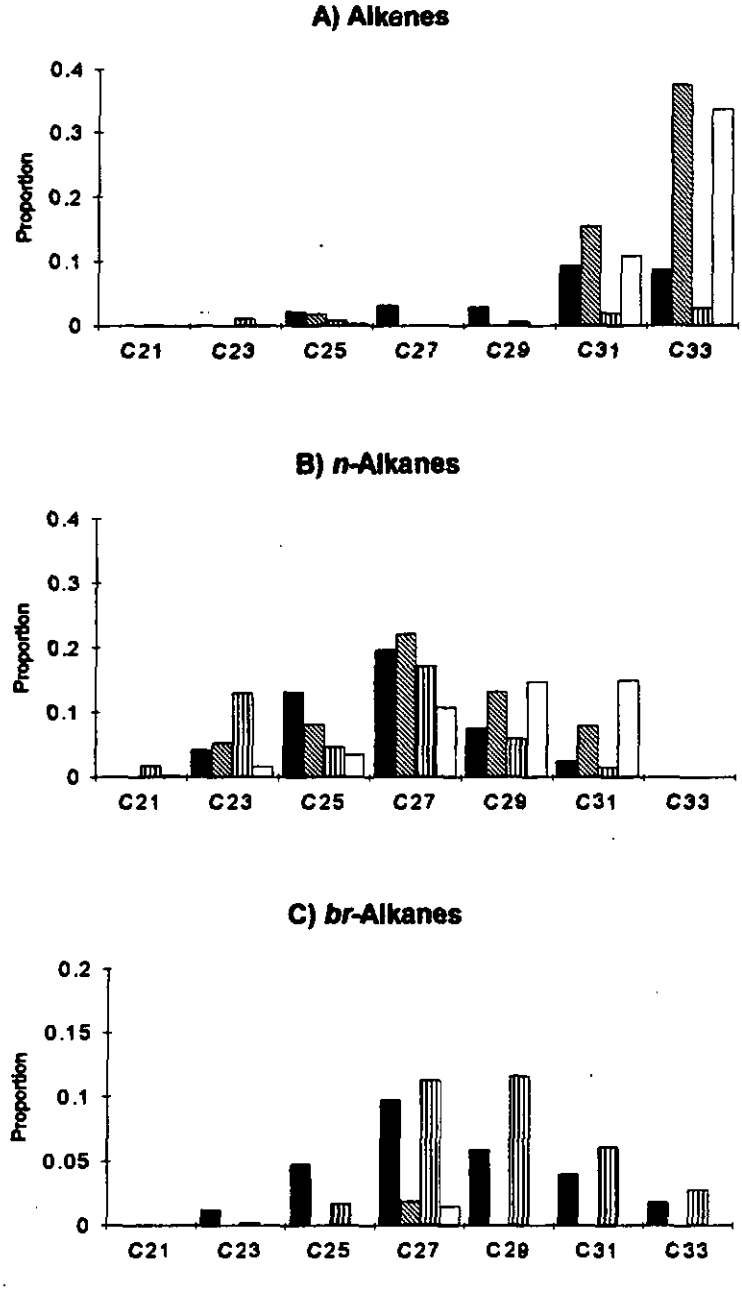

Figure 12: *Relative proportions A) of alkenes, B) of n-alkanes and C) of br-alkanes on cuticle of larvae (■), on walls of brood cells (▨), on newly emerged adult bees (▥) and on 6 day-old adult worker bees (□) (data of adult bees from Francis et al., 1989). Abscissa: chain length (number of C-atoms). Only compounds found in proportions of ≥ 1 % are drawn here.*

***Table 10:** Amounts of products found by GC-FID in raw hexane extracts of 8 day-old honeybee worker larvae (n = 5 extracts, each of 100 larvae, 15 min extraction with 50 ppm internal standard, only major compounds (≥ 1%) were included).*

	Weight (mean µg ± sd) per larva	mean (µg) per cm^2 of larval cuticle (approx. 2 cm^2)
Total weight	5.34 ± 0.80	2.67
saturated HC's	3.97 ± 0.60	1.99
n-alkanes	2.53 ± 0.47	1.27
br-alkanes	1.45 ± 0.17	0.73
unsaturated HC's	1.37 ± 0.24	0.69

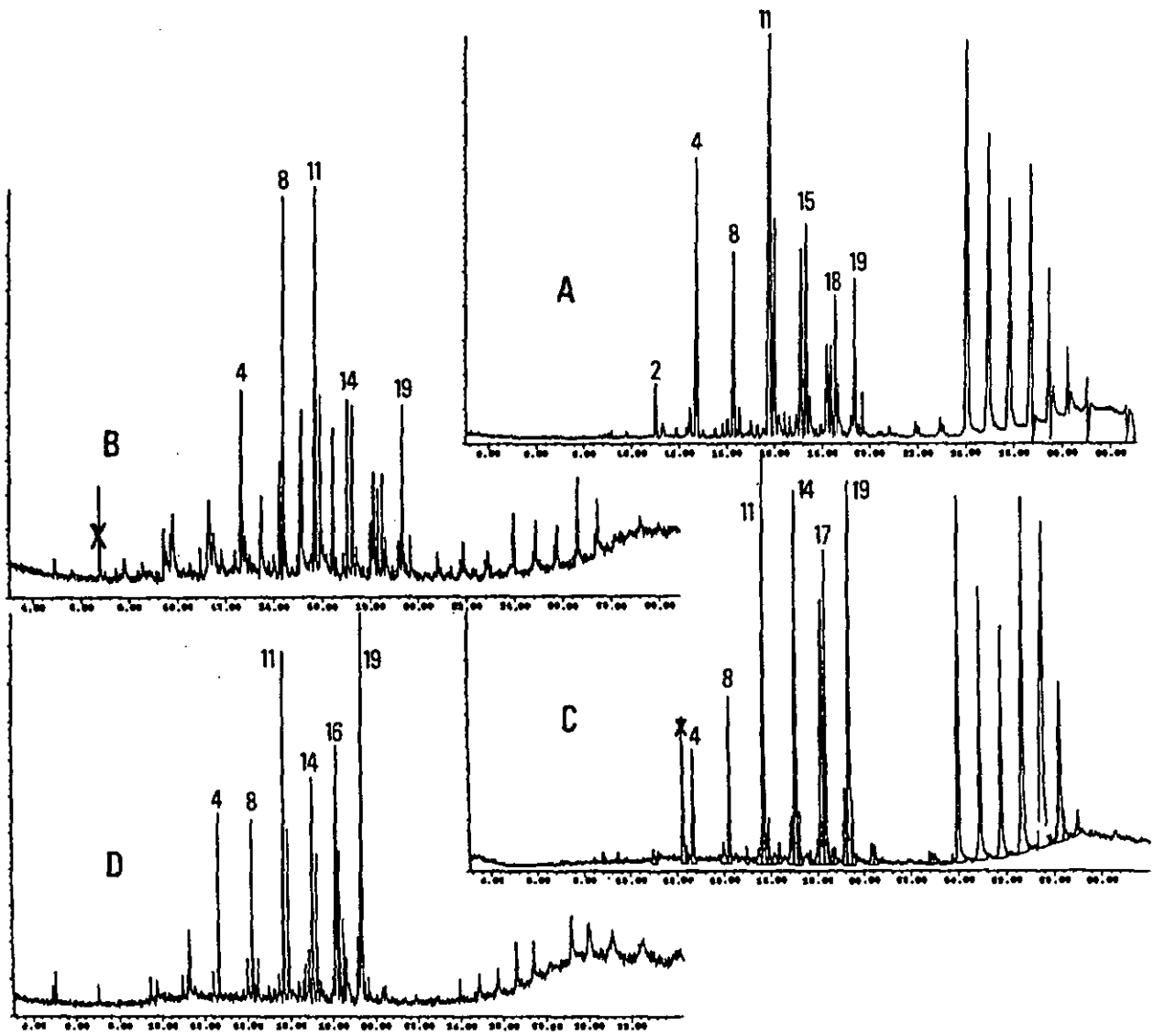

***Figure 13:** Chromatograms (GC-MS) of cuticle extracts of a) freshly emerged adult worker bees, b) of Varroa collected from (a), c) of mixed aged hive bees from the brood nest and d) of Varroa collected from these hive bees. X denotes artefacts (compounds derived from detergents used in glass washing). Identification of numbered peaks: see Table 9.*

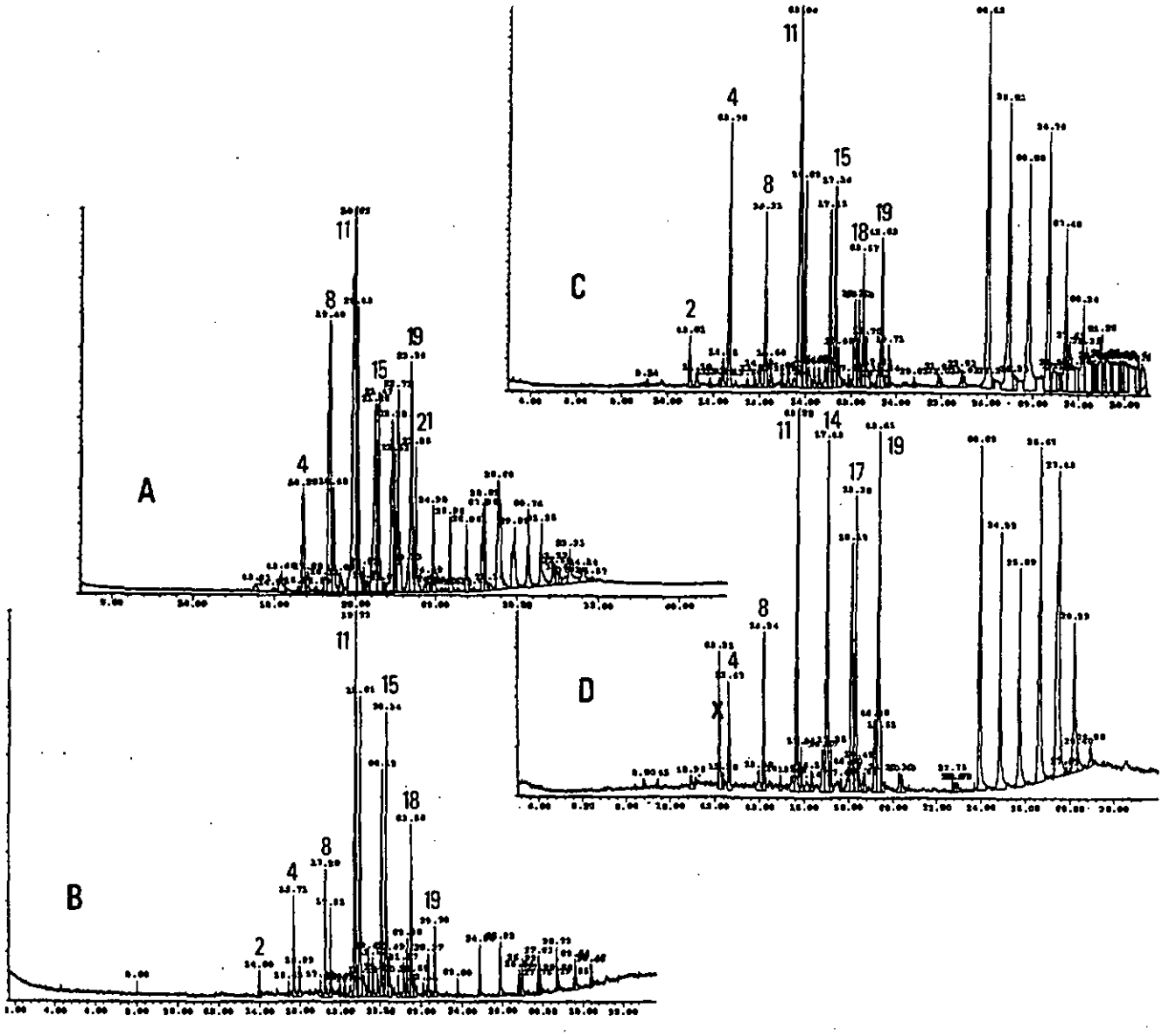

Figure 14: Chromatograms (GC-MS) of cuticle extracts of a) 8 day-old worker larvae, b) of 6 to 7 dey-old worker pupae, c) of freshly emerged worker bees and d) of mixed aged hive bees. X denotes artefacts (compounds derived from detergents used in glass washing). Peeks numbered as in Table 9. The starting temperatures were varied from 60°C for larvae, 70°C for pupae and 100°C for adult bees in these chromatograms so that the retention times of the compounds do not match.

5. DISCUSSION

5.1. Volatile stimuli on the servosphere

Palmitic acid (PA) was found to attract *Varroa in vitro*. However, PA is not specific for larvae since it also occurs on adult honeybee cuticle (Blomquist et al., 1980). It therefore remains unclear what stimulus allows the mites to recognize 8 day-old larvae.

Some 5 larva equivalents (leq) of condensate containing some 3 ng PA were attractive whereas a 2.5 µg-source of PA alone was necessary to elicit a similar response. This discrepancy in quantities of PA might be explained by the fact that some of the other compounds present in cold-trap extracts could reduce the sensitivity of the mite towards PA or that other constituents of the extract are attractive on their own. An attempt to verify one of the hypotheses by fractionation of the condensates resulted in the loss of activity in the bioassay. A different approach, i.e., gas-chromatography coupled with electrophysiology was explored, but unsuccessfully terminated.

Mean values and standard deviation may normally not be used to describe asymetric distributions as I did in describing the absolute values in deviation and turn angles. But since the sample was big enough (>2000 values) it became acceptable. However, the distributions of deviation angles shifting dramatically from a slightly preferred direction of 90° to the wind in humidified air to a clear upwind direction (0°) in presence of PA (Fig. 1 in chapter 4.1.) speak for themselves, and did not require any statistics. Statistical analysis of turn angles was made in using the normally distributed relative values (left/right shifts of the body axis).

"Ghost" peaks are known to occur in gas chromatography e.g. due to contamination by touching glassware or instruments with naked fingers where squalene, cholesterol and $C_{16:1}$, $C_{16:0}$ (PA) and $C_{18:1}$ fatty acids are deposited (Biedermann and Grob, 1991). The presence of squalene in the condensates hints to such a contamination because this precursor in steroid biosynthesis is not supposed to be synthetized by insects (Oberlander, 1985). The absence of cholesterol and fatty acids $C_{16:1}$ and $C_{18:1}$, however, contradicts such a contamination. Squalene could therefore originate from plant material, e.g. pollen which is known to occur in larval food jelly. Free PA was identified in extracts of adult bee cuticle (Blomquist et al., 1980) and detected in extracts of larva cuticle in this study where no squalene was present (chapter 4.2.). For this reason I may conclude that PA is in fact implicated in the attractivity of odour condensates of host larvae.

In addition, a mixture of *n*-$C_{25:0}$ and *n*-$C_{27:0}$ (1:1, in total amounts of 5, 50 and 500 µg) and the TLC subfraction F1A of larva cuticle extract (5 leq) had been tested on the servosphere but neither an upwind response nor arrestment of the mites was observed (results not reported here). I consider these tests as not satisfying because of the lack of a positive control (2.5 µg PA) in that test series. Thus, it is not proven that these compounds have *no* behavioral activity when presented as volatiles.

Only one other study exists to my knowledge on volatile chemostimuli involved in *Varroa*'s host recognition (LeConte et al., 1989). The authors found the odour of drone larvae active in a four-arm airflow olfactometer using a "gentle" wind bearing the chemostimuli. They identified three fatty acid esters (methyl palmitate, ethyl palmitate and methyl linolenate) as active constituents. The temporal secretion of these products was studied and a peak concentration at the time of operculation confirmed the suitability of the products for host recognition (Trouiller et al., 1991). In my study these products were either not found in odour condensates or at much lower quantities in cuticle extracts. Although our GC-MS analyses were qualitative, amounts of 50 to 200 ng *Varroa* attracting fatty acid esters per 8 day-old larva reported in Trouiller et al. (1991) should have given a far stronger detector response than we observed in our extracts. These differences can be explained by a partial hydrolysis of the esters in my study resulting in the presence of the corresponding fatty acids. However, PA was found in cuticuler extracts of larvae by Trouiller (1993) and in our own extracts of 8 day-old larvae (TLC fraction F7), and therefore it was not the product of an artefact which was tested on the servosphere. In addition, comparing the method applied by LeConte et al. (1989), i.e., delivering the odours in a gentle airflow and recording the animals' postition per time interval, with that in the study presented here, one cannot exclude that two different types of orientation responses were observed. There, mite arrestment might have occurred, while here the upwind response showed the attractivity of PA.

5.2. Contact-chemostimuli on the zoned membrane

Varroa was arrested on apolar fractions of cuticle extracts made from 8 day-old worker larvae. A single constituent of this active fraction, i.e., *n*-$C_{21:0}$ elicited the same response at amounts of ≥ 6 µg per cm^2. A mixture of synthetic *n*-alkanes C_{21} to C_{29} was active when containing 0.04 µg *n*-$C_{21:0}$ per cm^2 and subfractions F1A of the cuticle extracts arrested the mites at doses containing 0.02 µg *n*-$C_{21:0}$ per cm^2. Therefore, *Varroa*'s arrestment response is due rather to a synergistic effect of alkane mixtures than to a single constituent. Since singly tested *n*-$C_{23:0}$ to *n*-$C_{29:0}$ were not active, this shows that the arrestment behavior and border recognition is more specific than related to a fatty texture.

Neither PA, which was attractive to *Varroa* on the servosphere, nor the corresponding TLC fraction F7 containing free fatty acids nor MP nor TLC fraction F5 containing nonanel, fatty acid esters and fatty acids elicited the typical arrestment behavior of the whole extract including the recognition of the borders of the treated area. TLC fraction F5, however elicited longer stopping durations compared to the controls (notched box plots, chapter 4.4.6.), thus some of the products contained in that fraction may be involved in host recognition as suggested by LeConte et al. (1989).

The temperature at which the mites were held in the laboratory and at which the bioassay was carried out had a strong influence on *Varroa*'s walking behavior on the membrane. This can be explained by the fact that mites brought from 19 - 21°C ambient temperature to 32°C on the membrane un-

dergo an increase in temperature which has an activating effect. The increase is less pronounced when the mites were brought from 27°C ambient temperature to 32°C on the membrane and the mites therefore were less activated.

5.3. Cuticle hydrocarbons as semiochemicals

Saturated HC's occur not only on 8 day-old larvae, but also in washes of the walls of brood cells and on adult bees (Francis et al., 1989; own results in chapter 4.4.9). The discrimination between larvae and other sources of chemostimuli therefore cannot be based simply on the presence or absence of saturated HC's alone. The fact that *Varroa* differentiates between *n*-$C_{21:0}$ (active) and *n*-$C_{23:0}$ (inactive) proves the recognition of the chain lengths. In addition, *br*-alkanes in cuticle extracts of larvae elicited a walking behavior different from the solvent control. Both, proportions in HC chain lengths and proportions of branched and straight chains vary with host age (chapter 4.4.9) and therefore could provide discrimination cues (chapter 5.5.2).

HC's and especially saturated HC's have been reported to function as semiochemicals in numerous studies on arthropods. The tracheal honeybee mites, *Acarapis woodii* are arrested *in vitro* on a fraction of host cuticle extract containing saturated HC's suggesting an implication of these compounds in the recognition of young adult worker bees as hosts (Phelan et al., 1991). Straight chain HC's (uneven numbered C_{13} to C_{29}) have been reported as male sex pheromone constituents in the mites *Acarus immobilis* (Sato et al., 1993). The egg parasites *Trichogramma brassicae* are stimulated by uneven numbered C_{19} to C_{29} alkanes to oviposit into artificial host eggs (Grenier et al., 1993). Bumble bees, *Bombus terrestris* mark visited flowers with saturated and unsaturated C_{18} to C_{31} HC's from the tarsal glands (Schmitt et al., 1991). Honey bees defend their colonies against foreign bees. HC's are proposed to play an important rôle in the discrimination of nestmates from non-nestmates (Breed and Stiller, 1992). The gustatory discrimination between $C_{23:0}$, $C_{25:0}$ and mixtures of the two products by worker bees has been demonstrated (Getz and Smith, 1987). In addition, other social insects such as wasps, ants and termites seem to use HC's in nestmate recognition (Singer and Espelie, 1992 and ref. therein). Furthermore, workers of the ants *Camponotus vagus* discriminate between foragers and broodtenders (Bonavita-Cougourdan et al., 1993). The relative proportions of *n*-alkanes and of mono- and dimethyl alkanes (*br*-alkanes) on the thoracic cuticle of the two subcastes are significantly different.

5.4. Orientation mechanisms

5.4.1. Spontaneous anemotaxis of *Varroa* on the servosphere

Under constant wind conditions or when switching from one to a second airflow both of 0.2 m s^{-1} the rate of upwind turns shown by *Varroa* remains rather constant (9 to15 % responding mites; 10 s path segments). However, when the airspeed is increased from < 0.05 m s^{-1} to either 0.1 or 0.2 m s^{-1} then some 50 % of the mites show an upwind response. The upwind direction is not maintained in blank air as it is in air bearing PA (Fig. 2 in chapter 4.1.). Changes in windspeed alone eliciting

an upwind turning response is not a unique finding in arthropods (e.g. Heinzel and Böhm, 1989).

The orientation with respect to the wind (anemotaxis) is the basic mechanism of turning upwind and implicates the presence of mechanoreceptors in the case of *Varroa* which walks on a substrate (Schöne, 1983). Upwind responses to an increase of wind speed in the absence of semiochemicals prove the presence of such mechanoreceptors. The function of *Varroa*'s responses to changes in wind speed alone might be seen in such cases where the mites find themselves neither in the brood containing section within the bee hive nor on an adult bee, e.g. in the debris of bee colonies. Adult bees are most probably *Varroa*'s only means to regain the brood nest, i.e., mites which were taken from the debris of a hive stayed on the landing-board when deposited there, and climbed on passing bees, but did not walk into the hive (observation not reported here). An airstream created by a bee either passing by or ventilating could be recognized and used to orient toward such a bee.

5.4.2. Walking responses to contact-chemostimuli

The most obvious aspect of the mite arrestment on larva extract is the increased number of returns back onto the treated area after contacting its border. The majority of all returns observed on doses ≥ 1.2 leq were made while walking. *Varroa* might detect the border better while walking than when stopping since the drop in stimulus intensity per time unit, i.e., on arrival at the border, and the subsequent increase when returning to the treated surface is higher when moving. An integration of the stimulus intensity over time would represent a specific orientation mechanism, but at this moment the required information is not available to categorize this orientation process in a *taxis - kinesis* system (Schöne, 1983). In addition, *Varroa* most probably compare stimulus intensities at left and right P1 as demonstrated by directed turns upon one-sided loss of stimulation (see below). A combination of two types of orientation processes usually optimizes the orientation response. For instance, from a theoretical point of view an optimal orientation along a gradient should combine positive tropotaxis, direct orthokinesis and inverse klinokinesis (in: Schöne, 1983). The increased number of returns made while walking fits neatly into the general walking pattern of *Varroa*, where displacement activity is positively correlated with the amount of stimulus applied to the treated area.

In general, the mites walked faster and straighter on higher doses of the stimulus, showing a dose-dependent behavior when walking on a homogenously stimulating substrate. On patch borders they slowed down significantly but a dose-dependency in turn angles was not observed here. The turn angles observed at the borders were not significantly different from those on the treated area except for the dose of 1.2 leq. As long as turns made at the borders are directed toward the stimulus and not away from it (see below), the turn angles need not necessarily be greater at the border then on the treated area to bring the animal back onto the extract.

One may ask whether the increased walking speed on high doses of the stimulus is due to an overdose of HC's presented to *Varroa*. Because the mites walked similarly at the borders of areas treated with 1.2, 2.9 and 5.9 leq (Tables 5 and 6, chapter 4.4.7) overdosing seems not very likely in this case.

Varroa might be capable of effecting corrective action at the border when one leg 1 alone looses contact with the stimulus - provided it perceives the relevant chemical information at P1. To test this hypothesis I analyzed all returns made during 5 min test runs on 5.9 leq where the mites approached the border at angles of 60° or lower, i.e., where I could assume with some assurance that the mites had first touched the border with just one of its P1. In 85 % of 144 cases, turns following border contact were made towards the extract and in only 15 % away from the extract. These numbers are well removed from an equal distribution (Fisher-Exact, $p < 0.001$). This allows one to conclude that the mites do not change direction at random on stimulus loss but directed towards the stimulus treated substrate, suggesting unilaterel loss of stimulation at P1 and correction towards the still stimulated side.

5.4.3. Analysis of *Varroa* behavior at the borders of an area treated with a contact-chemostimulant

Mites returning after a border contact back onto the treated area do so with significantly dose-dependent angles to the border tangent: the higher the dose, the smaller the angles observed. Similarly, on higher doses smaller arrival angles are recorded. How could this dose-dependancy of the angles to the border tangent, especially of the departure angles, be explained ? When testing the hypothesis that the underlying mechanism could be a billard ball effect, i.e., that the angle of arrival equals the angle of departure, we detected no significant correlation between arrival and departure angles on 5.9 leq F1A.

However, the sums of arrival plus departure angles, *i.e., the correction* angles for returns within 0.2 s on 5.9 leq F1A were analyzed and a significant correlation with the arrival angles was observed. Of course, a high degree of autocorrelation is to be expected here, i.e., correlation of factor A+B (arrival + departure angle = correction angle) to factor A (arrival angle) if factor B is constant or linearly related to factor A. The observed r-value for the correlation between the arrival angle and the correction is 0.874. Since it is close to 1 this means that the size of the correction angle is about 75% ($r^2 = 0.76$) due to factor A, the arrival angle. For returns after a longer duration at the border - with a maximal displacement of 3.4 mm - this correlation was lower but still significant, as it was on lower doses of extract except for 0.2 s contacts on 1.2 leq.

In the following paragraphs I try to explain the dose-dependent departure and arrival angles in a *post hoc* treatment. A detailed analysis was not possible because the raw data, i.e., the video-protocols were not of sufficient quality. The results therefore should be treated cautiously.

Dose-dependent departure angles are most likely connected to the perception of stimulation. An

explorative approach assumed that firstly the mites exhibit a dose-dependent walking behavior when moving on a homogenously stimulating substrate (as demonstrated in chapters 4.2. and 4.4.7). On loss of stimulation, such as at the border, normal behavior is interrupted and substituted for by turning to re-establish full contact with the stimulus. The mite will undertake only the necessary shift in the direction of displacement to bring itself back into contact with the adaequate stimulus. As demonstrated above, the stronger the stimulation on the treated area, the lower the angles of arrival at the border, and consequently, smaller turns are required to bring the animal back onto the extract. Since the mite walks straighter on an area treated with an adaequate stimulus, the arrival angles at the next border contact are low and subsequent corrections to regain full contact are low.

If the mite turns at the border only as much as necessary to re-establish full stimulation, then the departure angles required for this correction in the model are uniformly low for contacts with the border involving one P1 alone and with only low variance for contacts with the border involving the palps *irrespective* of the arrival angle. Thus, the departure angles to tangent at the border can be considered a constant. And, as shown above, a constant added to the arrival angle must result in a high correlation between arrival and correction angle. The observed correction angles for returns within 0.2 s fit into the range delimited by the model for arrival angles of < 60°. However, the mites correct their walking direction more than predicted by the model for angles > 60°. The angles to tangent recorded here were made over 0.6 s intervals before and after border contact. A mean turn angle of some 20° was observed per 0.2 s interval. (chapter 4.4.7.). Thus, some of the deviations of the observed correction angles from those predicted by the model may be due to turns actually made but smoothed out in the 0.6 s arrival or departure vectors.

The video-protocols do not permit testing the hypothesis that turns at the borders are just enough to re-establish full stimulation, mainly because under the given experimental conditions the location of the border on the underlying ink circle cannot be pinpointed with the required precision. But another way of testing the hypothesis mentioned above was explored. The ring of treated substrate has an inner and an outer border (Fig. 15). If the hypothesis is true then we expect smaller correction angles on the inner convex than on the outer concave border for similar arrival angles because the stimulating surface covers more than 180° at any point on the inner but less than 180° on the outside border. Plotting the correction angles for inner and outer border contacts separately with the respective arrival angles provided in fact lower regression lines for the inner contacts, and the means of the correction angles were systematically lower on the inner border of each dose than on the outer border. Although the differences between the inner and outer borders were not significant, these findings represent a tendency which substantiates the above mentioned hypothesis.

The mites may discriminate better between a stimulating and a non-stimulating substrate at the borders of high doses than of low doses if - as it appears - they are able to perceive the quantity of TLC subfraction F1A applied to the membrane.

Better discrimination by the moving animal may result in the perception of loss of stimulation at bigger distances between the centre of gravity of the mite and the sensory structures already on the border, i.e., already after contacting the borders with P1. Thus, the initiation of the turn may be dose-dependent, i.e., upon contacting the border with one P1 only or subsequently with the palps, and therefore determine to a high degree the departure angle in our model. In addition, the distance covered per 0.2 s increases in a dose-dependent fashion (chapters 4.2, and 4.4.7). The faster the mite hits the border the steeper the drop in stimulus intensity per time unit. Thus walking fast - within limits - could tend to highten the animal's recognition of the border of an adaequate stimulus and contribute to tha dose-dependency of angles made at the borders of TLC subfraction F1A.

The low departure and arrival angles lead to tracks close to the outer borders of the circular treated arena (Fig. 15). Functionally, this may respresent a mechanism to search for the location of a "subsequent" stimulus located at the border of an area containing the stimulus, i.e., the cleft where the bee larva meets the cell wall, in pursuance of a behavior cascade (as outlined in the chapter 5.7.1.). Furthermore, the knowledge of such a behavior is important when designing future experiments, especially experiments to study discrimination between two simultaneously presented contact-chemostimuli.

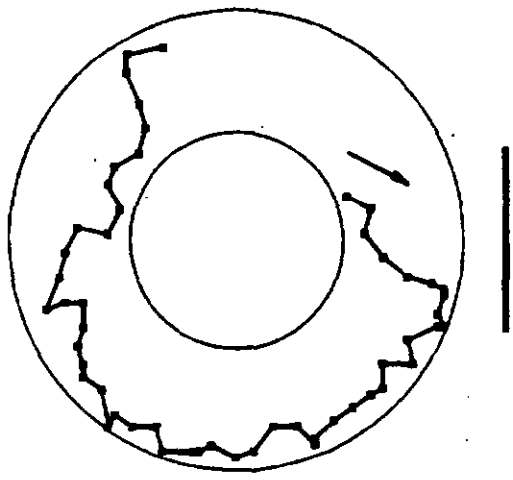

Figure 15: Segment of path made on a circular arena treated with 30 µg n-$C_{21:0}$ cm^{-2} demonstrating how low departure and arrival angles lead to a walk close to the borders with occasional bigger turn angles. Arrow: walking direction; duration of segment shown is some 10 s. Bar represents 10 mm.

5.4.4. Responses to combinations of stimuli

Chemostimuli often need the combination with other cues to elicit specific responses (chemostimuli of parasitoids reviewed by Vinson, 1976). HC's may carry different information for the mites when encountered on a humid flexible substrate as with larvae than on a relatively dry and hard surface such as comb wax. Ralative humidity also modulates the responses of *Ostrinia nubilalis* males to the female sex pheromone by increasing the in-flight arrestment with increasing humidity (Royer and McNeil, 1993). Pheromone releasing females aggregate in high-humidity sites in this lepidopteran species. An interference of the substrate quality, i.e., glass, filter paper and biological membrane with the mite response to total cuticle extract of 6 day-old larvae was observed in this study. The subsequently demonstrated arrestmant behavior was shown only on membranes. Another example for such an interferance (cross-channel potentiation, Bell, 1990) is in *Varroa*'s upwind walking in presence of PA on the servosphere, where only the combined mechano- (wind) and chemostimulation (PA) elicited the orientation response.

5.5. Chemooriantation of *Varroa*

5.5.1. Chemosensillae of *Varroa*

Adult female *Varroa* bear chemo-sensitive sensillae in a shallow pit situated dorsally on the tarsus of P1 (Fig. 16a) and at the distal end of the pedipalps (Fig. 16b). On P1, sensillae were observed with pores on the shaft suggesting an olfactory function, as well as terminal pore sensillae, which are usually believed to be used in contact-chemoreception (Milani and Nanelli, 1988). On the palps only terminal pore sensillae were identified, but olfactory sensillae also may be found there (Liu, 1990). In addition, a ring of sensillae around the pit

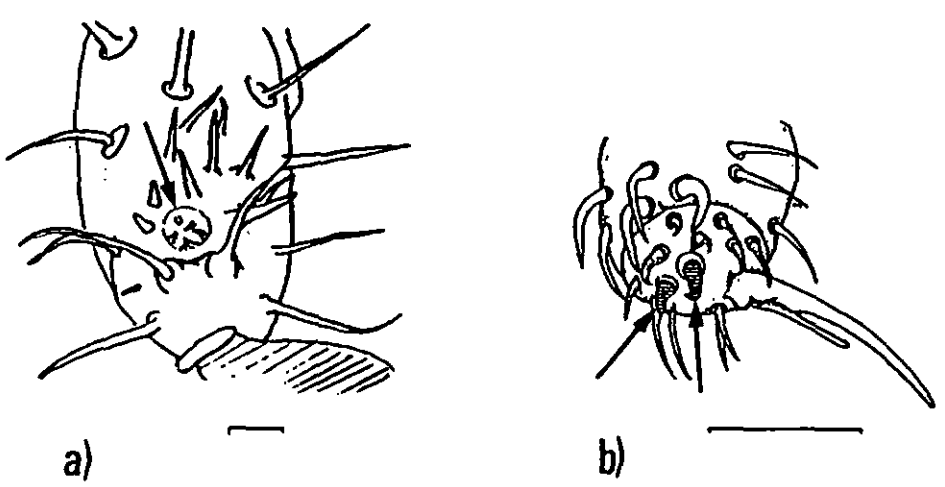

Figure 15: Body parts of adult female Varroa *bearing chemosensitive sensillae. a) dorsal view of P1 with tarsus and pretarsus, the latter shaded. A pit (arrow) contains 9 sensillae which are probably involved in olfactory and contact-chemoreception, and in thermo-and hygroreception. b) ventral view of the distal end of the pedipalps showing two terminal pore sensillae (arrows). Bars represent 20 µm. (re-drawn a) after Milani and Nanelli,1988, and b) after Liu, 1990)*

on P1 contains sensory hairs; some of them appear to be contact-chemosensillae (Ramm and Böckeler, 1989). Furthermore, mechano-sensillae are most probably present on P1 and on the palps. *Varroa* does possess hairs on the ventral side of the tarsus of P1, some of which may be chemosensory.

5.5.2. Cell invasion: chance or attraction ?

Mites are found at the cell base of only worker cells which will be sealed within the next 20 h (Boot et al., 1992b). Two hypotheses have been proposed to explain this observation. First, the mites try any cell containing a larva (Ifantidis, 1988). As 8 day-old larvae fill the cell base completely, the mites first have to squeeze between larval body and cell wall when walking to the cell base (Fig. 17). Mites brought into contact with food jelly are rendered immobile (Rath, 1991), and therefore stay at the cell base, trapped until the larva consumes the food after the cell has been sealed. However, the question remains open, as to how the mites select their host and what actually attracts them towards the cell base. Food jelly may contain an attractant but I am not aware of any study treating this topic. Secondly, mites are attracted specifically to 8 day-old worker or 9 day-old drone larvae (LeConte et al., 1989). In both cases chemoorientation is most probably involved in the cell invasion, i.e., either as contact-chemoorientation in host acceptance or as olfaction in host location (terms following Vinson, 1976). This is even more probable when considering that *Varroa* lives in blindness, i.e., on the one hand it livas within the dark bee hive among densely clustered bees or within a sealed brood cell, and on the other no indication of *Varroa* sensibility for light was found in literature.

A nurse bee depositing food at the base of brood cells inserts its head and thorax into the cells (Lindauer, 1953). Mites on the thorax were pushed backwards by the cell's rim and could not leave their nurse bees within the brood cells (occasional observations). On such occasions mites may perceive chemicals released by or associated with the larvae. The mites leave the bees outside the brood cells (Boot et al., 1992a), so they must walk at least from the rim of the brood cells to the cell

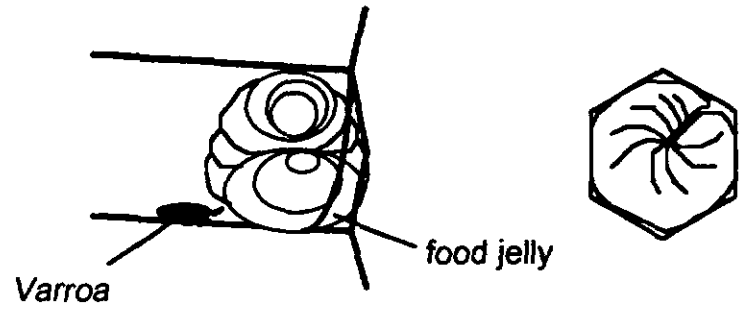

Figure 17: Schema of an 8 day-old larva filling the cell base (left: longitudinal section; right: view from open top of the cell). Such larvae were used for harvesting chemostimuli in this study. Mites squeeze between cell wall and larval body to reach the food jelly. Since Varroa *is found there with its legs facing the larva, it previoulsy must have been walking on the host.*

base - an argument in favour of an age-specific attraction by volatile cues. All mites we found until now in the food jelly had their ventral sides facing towards the larvae. Once below the larvae the mite cannot turn, thus indicating that the mites were walking on the larvae and not on the cell wall prior to reaching the cell base. Therefore they systematically switch from the cell wall to larva during cell invasion. *Varroa* is most probably capable to discriminate between wax and larva using chemostimuli.

5.5.3. Cues available to *Varroa*: surface products of immature and adult bees and of comb wax

GC-MS and GC-FID analyses of extracts of 8 day-old larve, 6 to 7 day-old pupae, adult worker bees and comb wax indicated both, the occurrence of saturated and unsaturated HC's common to all extracts and some differences between the solutions analyzed. Shifts were noted from saturated to unsaturated, from branched to straight and from shorter to longer HC chains with increasing age. Comb wax had a HC profile relatively close to adult bee cuticle. This part of the study gives only a first and mostly qualitative insight into the chemical environment and thus to the chemostimuli available to *Varroa*. A detailed study on this topic should include more sophisticated methods in the preparation of the extracts, i.e., extraction of individual hosts, the monitoring of the separation steps, i.e., recovery of treated material, and the quantification of a sufficient sample size to allow statements substantiated by statistical cluster analysis (e.g. as in Smith, 1991). The main goal of my study, however, was to determine the components eliciting *Varroa* responses to host products *in vitro*.

In the study presented here, chemical analyses had been made on a rough time scale including one larval, one pupal and two adult life-stages of worker bees. A detailed study dealing with the changes in cuticle HC's of larval and pupal honeybees is unknown to me. On adult worker bees, proportions of *n*-alkanes increase, those of *br*-alkanes decrease while the proportions of saturated HC's decrease and those of unsaturated increase with age, the strongest changes taking place within the first 6 days after emergence (Francis et al., 1989). Continuous changes in *Apis* development would be different to the changes reported for the wasp *Vespula germanica* where HC profiles from larvae and adults resemble each other more than those from pupae (Brown et al., 1991). The results presented here confirm data on the ontogeny of adult worker cuticle HC's (Francis et al., 1989), namely the high proportion of *n*-$C_{23:0}$ on newly emerged bees and the marked increase of the alkenes C_{31} and C_{33} in cuticle extracts of adult bees compared to larvae or pupae.

Marked differences have been observed when comparing the HC profile of *A. mellifera* workers to that of *A. cerana* workers (Francis et al., 1985). In *A. cerana*, as in *A. mellifera*, *n*-$C_{27:0}$ makes up the greatest proportion of cuticle extracts of workers of random age, but *n*-$C_{25:0}$ is higher in *A. cerana* and the other *n*-alkanes lower. The proportions of the *br*-alkanes are similarly low in workers of both species. The alkenes $C_{31:1}$ and $C_{33:1 \text{ and } 2}$ are present at far lower amounts on *A. cerana*, e.g. $C_{31:1}$ was not detected in *A. cerana* but at 9-15% in

A. mellifera. Similar, comb wax of *A. cerana* is composed of a reduced number of major components compared to *A. mellifera* comb wax (Tulloch, 1980). Data on A. *cerana* larvae are unknown to me. If the same tendencies during the honeybee development occur in *A. cerana* as in *A. mellifera* where the proportions shift as mentioned above, then - due to the reduced number of major components - one would expect greater differences between larvae and adult bees or comb wax in the case of *A. cerana* than *A. mellifera*. The mites in *A. cerana* colonies would then have a different set of discrimination cues at their disposition. However, the alkanes C_{19} to C_{29} found active in the study presented here are present on *A. cerana* workers and their comb wax confirming the hypothesis that any chemostimuli of importance concerning host recognition should be available to the parasite in colonies of the primary host.

5.5.4. Discrimination of chemostimuli

For both, volatile and contact-chemostimuli found active in bioassay, no specifity to larvae was found. Thus, these compounds would not permit by their mere presence or absence discrimination between different sources and consequently are not alone responsible for host recognition. *Varroa* may discriminate between sources using a hierarchy of stimuli from unspecific to more specific cues. Such a hierarchy was as proposed for honeybees, where nestmate discrimination is suggested to follow priority levels (Breed and Glennis, 1992). For instance, the presence or absence of a cue has first priority. If it is present, then more specific cues such as relative proportions are checked for further discrimination. It is tempting to speculate on the information content of the chemostimuli for *Varroa*, where the omnipresent PA and *n*-alkanes may respresent cues of a high priority but a low source specifity. PA and *n*-alkanes may convey the information "now I am inside a bee colony" to the mites. The absence of these products consequently may mean "outside of a bee hive", and in such a case any further source discrimination in the context of cell invasion is meaningless to the mites. Internally branched alkanes found in higher proportions on immature honeybees (Nation at al., 1992; own results in chapter 4.4.9.) and fatty acid esters with peak concentrations on the cuticle of larvae at operculation (Trouiller et al., 1991) have a higher source specifity. Thus, these products - or some of them - may contribute to the source discrimination on a lower priority level, especially because they elicited either higher walking (*br*-alkanes) or stopping durations (TLC fraction F5) in the membrane bioassay.

The composition of the food jelly varies with the age of the recepting larvae in the relative proportions of moisture, protein, sugars, lipids and free amino acids (Brouwers et al., 1987). This shows that the nurse bees assess the age of the larvae by volatile cues as suggested by Huang and Otis (1991b) or by contact-chemostimuli as proposed by Free and Winder (1983) in order to deposit the appropriate jelly. The same cues used by bees in larval age recognition could serve *Varroa* for host recognition, i.e., two of the *Varroa* attracting fatty acid esters were identified in a blend of similar esters which induced the cell capping behavior of nurse bees (LeConte et al., 1990). In addition, food jelly *per se* could be a cue for age discrimination

by *Varroa* since its composition is correlated with the host age.

In addition, the same HC's which were identified as chemostimuli on the cuticle of larvae in this study have been found on *Varroa* cuticle (chapters 4.4.9. and 5.7.3.). These products could well serve the mites as intra-specific semiochemicals, especially in such cases where more than one mother mite reproduce in the same brood cell (Donzé and Guerin, 1994).

In an experiment not reported here I took mites from the debris of heavily infested bee colonies and deposited them on the landing board of a bee hive. There the mites had all stimuli emanating from a bee colony at their disposition, and could have walked towards them. However, the mites remained at their position and climbed on adult bees, when possible. Thus, the results of the *in vitro* tests might reveal stimuli not just active in the search for larvae but also for adult bees. However, the behavior of the mites on the landing board differed strongly from the behavior observed on membranes coated with saturated HC's in the bioassay of contact-chemostimuli. On the landing board the mites stayed with only occasional locomotion and with their P1 raised. On the contrary, they showed prolonged walking activity on the HC treated membrane with their P1 contacting the substrate regularly. Thus, the mites were clearly differently reacting to stimuli on the landing board (probably absence of *n*-alkanes as contact-chemostimuli, presence of other stimuli is unknown) and to those on the membranes (presence of contact-chemostimuli). On the servosphere the observed behavior in wind bearing PA differed from that shown on the landing board in the prolonged and upwind directed locomotion while the P1 were mostly raised.

5.6. Other stimuli

Short-distance-attraction is suggested by another study (Ghzawi/Liebig pers. comm.) in which mite movement was studied in a bee hive containing a section with brood and nurse bees and a second with *Varroa*-infested bees. Mites moved from the brood-free section to the brood, if the distance between the sections was 15 mm or less. The mites stayed on the bees if the larvae were younger than 8 days, but invaded cells occupied by 8 day-old larvae. Stimuli associated with 8 day-old larvae therefore are involved in an age-specific attraction of the parasite towards its host. However, the stimuli may be indirectly associated with the larvae and may even originate from the nurse bees' behavior tending brood. Mites invading cells 20 h before sealing prove that the invasion is not directly linked to the bees' capping behavior.

Mechanostimuli have been proposed since artificially shortened brood cells where larvae were closer to the cell's opening had a higher infestation rate than natural cells (Goetz and Koeniger, 1993). However, the mites would be closer to the larvae at the rim of shortened cells, although at least 2.5 mm too far away to touch them, and so perceive a chemostimulus at a higher concentration - assuming a concentration gradient of the signal within the cell. Thus, these results hint rather to chemostimuli. (Bees regulate the hive atmosphere by fanning and thereby create air currents. The interior of open brood cells may be protected from

such currents, but molecules moving out of the cell may be rapidly diluted and dispersed). Temperature may be involved since brood is generally warmed up to 34 - 36°C. The mites have a thermopreferandum between 32.6°C (LeConte and Arnold, 1987) and 34°C (Pätzold and Ritter, 1989), and *Varroa* responds to temperature differances of 1.1°C (LeConte and Arnold, 1987).

5.7. Behavior during cell invasion

5.7.1. Sequence of behavior elements

An underlying hypothesis of this study is the existence of a sequence of behavioral acts each elicited by the proper stimulus. Such behavioral cascades have been widely described in arthropods, e.g. for host finding in parasitoids (Vinson, 1976) and mites (Egan, 1976), or egg laying in moths (Ramaswamy, 1988). An attempt to confirm or reject the existence of a behavioral sequence by directly observing the cell invasion failed. Therefore a behavioral cascade in *Varroa* host selection remains an assumption. It is an assumption to which I found no contradictory evidence. Rather, the inactivity of the attractants PA and MP when the mite could contact them tends to confirm the assumption.

Cell invasion as a behavioral sequence may contain the following steps. First, the mites stay between the intersegmental folds on adult hive bees. Functionally the mites may there either hide from the worker bees and/or they may require the period on adult bees for maturation (sperm maturation in females takes 2 to 4 days after mating; Hermann, Donzé, Bachofen, pers. comm.). It is unknown to what cue(s) the mites respond when they leave the intersegmental folds - it could be a change in the cuticle HC profile of the bees or, e.g., a hormonal stimulus produced by either the bees or the mites. By leaving the intersegmental folds of the worker bees the mites may uncover receptors hitherto covered by the tergites or sternites of the bee to scan their environment. Since the parasite is moved by the bee through the environment this stage may serve as ranging (terminology according to Bell, 1990), i.e., moving until the proper stimulus for local search is perceived. On the perception of such a stimulus the mites would descend from the worker bee to the brood comb. Only vague hints exist as to what really happens such as "adult bee had to come close to a brood cell before the mite invaded" (Boot et al., 1992a), i.e., whether the mites enter just the next cell or move over more than some body lengths and whether they walk at random or directed is unknown. Similar, it is not known whether the mites respond to directional cues or to non-directional stimuli within the hive. However, *Varroa* is capable to respond to directional cues as evidenced by the responses to PA on the servosphere (chapter 4.1). Host acceptance may happen during contact with the larval cuticle, upon which the mite may search for the cleft between larval body and cell wall.

Usually parasites/parasitoids search for a specific site within a kairomone patch e.g. host eggs or larvae for oviposition or feeding (e.g. Zhang and Sanderson, 1993). Paths made by *Varroa* show a striking similarity to those made by the egg parasitoids *Nemeritis canescens* (Waage, 1978) on a patch of host kairomone. But *Varroa* increases its walking speed on host kairomone while *N. canescens* walks slower. The mites may respond to

cuticle extracts of larvae with the search for the next stimulus in the behavioral sequence of cell invasion. This may be presented at the place where the larval body meets the cell wall, a place which is not a point but the boundary of the open larval surface and the cell wall. Here mites may sqeeze between the larva and the cell wall to reach the larval food jelly.

Crawling into this cleft until the food jelly is reached would represent the last step of the cell invasion. It probably is induced by thigmotaxis. Some 20 h later, the mites show no more thigmotactic behavior when liberated from the larval food in the sealed cell (Donzé, pers. comm). Thigmotaxis therefore may be induced by stimuli present 20 to 0 h (worker larvae) and 20 to 40 h (drone larvae) before, but absent during cocoon spinning after operculation of the brood cell. In addition, the mites may be attracted to the cell base by the food jelly. Apart from a note hinting to akinesis of the mites induced by food jelly (Rath, 1991), the latter has not yet been studied as a source of chemostimuli. The larvae fill the lower part of the cell completely when *Varroa* invades brood cells (Fig. 17), which suggests an olfactory function of the jelly - if any at all. However, the time spent immobile beneath the larvae until the cell is sealed and/or the food jelly is consumed could be interpreted as an adaptation to seek shelter from worker bees in *A. cerana* hives, either by being covered by the larval body or by being imbibed in the food jelly. In addition, it could synchronize the mite oogenesis to host age. *Varroa* punctually lays its first egg some 60 h after operculation in both worker and drone cells (Donzé and Guerin, 1994).

Calis et al. (1990) report on a fairly constant daily fraction of the mite population of a hive on adult bees as invading brood cells. They conclude that under the given experimental conditions it was rather colony-dependent, i.e., the ratio of adult bees to suitable brood cells, than mite-dependent factors as such which determined the rate of invasion. This is to say bees had to bring the mites close to the brood cells. But other factors most probably determine the timing of cell invasion, such as the age of emerging mites (earlier or later daughter) and sperm maturation in these females. Egglaying in *Varroa* is well coordinated with host development (Donzé and Guerin, 1994), thus cell invading mites must be in the physiologically appropriate state which allows them to follow bee development.

5.7.2. Risk-minimizing hypothesis

If a host develops an efficient defense behavior against its parasite then - in the case of a coevolution of host and parasite - it should not surprise us if the parasite develops mechansims to combat host defense (Kim, 1985; Janzen, 1985). Although helminth parasites induce immune responses in man they protect themselves by a thick extracellular cuticle and/or by antioxydant enzymes present on or secreted by their cuticle (Maizels et al., 1993). Another well known example of host response avoidance is by *Trypenosoma* sp. which change their cell surface antigens to evade recognition by antibodies released by the host immune systeme. The asian bee *A. cerana* is capable of detecting *Varroa* mites in worker brood cells as well as on adult workers, and can remove them from the hive (Peng et al., 1987, Büchler et al.,

1992, Tewarson et al., 1992). Such a detection capability on the part of the host means a risk for the mites which expose themselves to the bees when walking on combs during the process of brood cell invasion. Selecting specifically brood cells of 20 (worker) or 20 to 40 h (drone) before operculation may reduce the exposure time to the bees, thus supporting the hypothesis of specific attraction to the appropriate host stage.

A larva is visited by nurse bees with an age-dependent frequency, reaching a peak frequency some 24-12 h before operculation (Brouwers et al., 1987). A nurse bee feeds larvae at a frequency of 1-2 times per h (Lindauer, 1953). Since feeding occurs only in about one sixth of all visits (Huang and Otis, 1991b), a nurse bee inserts its head and thorax up to 10 times per h into a brood cell occupied by a larva. *Varroa* prefer nurse bees over freshly emerged workers or forager bees after emergence from the brood cell (Kraus et al., 1986; LeConte and Arnold, 1987; Steiner, 1993). Mites carried by such bees are brought very close to host larvae. The mites therefore don't need to to range in the search for suitable brood cells, thus can save energy and minimize the risk of being detected.

Optimal risk-minimizing for *Varroa* should include as few and as short dislocations as possible on the part of the mite. However, one can observe *Varroa* mites running over the surface of combs when opening heavily infested bee hives. Or, mites may leave their host bees when held in the laboratory as in this study. These observations of mites leaving their hosts with apparently no goal in the immediate vicinity seem to contredict the proposed risk-minimizing hypothesis. In both cases mentioned the naturel conditions of bee hives are heavily disturbed, i.e., opening bee colonies usually is prepared by pumping smoke into the hives and in the laboratory only small groups of adult bees (up to 100 individuals in this study) were held in a large volume containing no beeswax. Therefore, observations of *Varroa* leaving their host bees in these cases do - in my opinion - not mean that such events are normal in undisturbed bee colonies. However, the only way to confirm or reject the hypothesis of risk-minimizing behavior is by direct observations in the bee hive. An attempt to observe *Varroa* cell invasion *in vivo* was abandoned due to technicel difficulties.

Some conclusions drawn here are only valid if the mites show the postulated risk-minimizing behavior. If this is not the case, then a specific attraction to the appropriate host life-stage is not required. Host recognition then could be based solely on contact-chemicals. For instance, shortened brood cells could then be infested at higher rates because larva seeking mites would meet the larvae with a higher probability in shorter than in longer cells.

Virtually no mites are found in worker brood cells of *A. cerana* which might be due to removal of mites from such cells (Rath and Drescher, 1990). Risk-minimizing here would mean for the mites to invade drone cells only. Therefore, one could expect sex-specific stimuli for host recognition of *Varroa* in *A. cerana* colonies. Drone cells are generelly built at the periphery of the brood nest where in addition to chemostimuli, lower temperature and humidity might serve as cues. *A. mellifera*

drone cells have a 8-9 times higher infestation rate than worker cells (Schulz, 1984). The perferance for drone brood is maintained even when the drone combs are placed in the center of the brood nest (A. Imdorf, pers. comm.). The high infestation rate of drone cells in *A. mellifera* might be caused here either by a higher attractivity due to qualitative or quantitative differences of larvae, cell wax or food jelly (LeConte et al. (1990) reported higher amounts of the *Varroa* attracting fatty acid esters present on drone than on worker larvae) or by longer periods of drone cell exposure to mite invasion (Boot et al., 1992b).

5.7.3. Adaptation to host defensive behavior by mimicry: Cuticular hydrocarbons of *Varroa* and its host

Higher proportions of *br*-alkanes were present on both, freshly emerged workers and their associated mites than on hive bees of different age. A close fit between cuticle of host and parasite was observed for the alkenes C31:1 and C33:1 and 2. Nation et al. (1992) showed a striking similarity between *Varroa* and host cuticle constituents, for alkenes and *br*-alkanes, when comparing extracts of individual pupae, adult worker and drone *A. mellifera* and their *Varroa* parasites. Our results confirm this observation in general, but with two exceptions. First, *br*-alkanes on mites of mixed aged hive bees were found in higher proportions than on their hosts. Secondly, *n*-C23:0 was present on mites from freshly emerged bees in a lower proportion than on their hosts. These differences could be due to the fact that in the study presented here mites and bees were pooled for extraction, whereas there they were extrected and analyzed individually. Or they could originate in a biosynthesis of the HC profile by the mites, which would lead to the imitation of host cuticular HC's some time after the infestation of the actual host.

The basis of *A. cerana*'s defensive actions against the mite (Peng et al., 1987; Büchler et al., 1992) must be a recognition of the parasite by the bee. Surface chemicals of the mites' cuticle most probably are involved in the detection of mites on adult workers, although the basis of detection of the parasite within brood cells remains to be clarified. The above mentioned similarities of host and parasite HC profile may well serve to reduce detection by the host. Peng et al. (1987) harvested *Varroa* from *A. mellifera* colonies which carried most probably substantial amounts of C31:1 (Nation et al., 1992 and my own results) whereas *A. cerana* workers have almost no C31:1 (Francis et al., 1985). These C31:1 bearing mites were detected and removed by *A. cerana* workers (Peng et al., 1987). *A. cerana* workers detect mites originating from *A. mellifera* colonies better than mites from *A. cerana* colonies (Tewarson et al., 1992), a result which lends credence to the idea that *Varroa* mimics its host's cuticle HC profile. Similarly, ant parasites, *Microdon* sp. use *n*-, *br*-alkanes and alkenes to simulate the cuticular HC profile of a specific host stage, i.e. pupae (in: Dettner and Liepert, 1994). Imitating the host's HC's profile therefore might be of extreme importance to the survival of *Varroa*.

In the above paragraph the function of the similarities between *Varroa* and *A. mellifera* cuticle HC's was interpreted in the context of the defensive behavior of *A. cerana*, i.e., detection and removal of

the parasite. Although some aspects of a defensive behavior have been observed in *A. mellifera* (Peng et al., 1987), *Varroa* is not exposed here to a similar pressure as in *A. cerana* which is proven by the fact that mite populations reach far higher densities in *A. mellifera* causing colony collapses.

5.7.4. Behavioral adaptation to host defense

Chemical camouflage can only protect the mites in chemically stable surroundings or in an environment which at least changes slower than the mites' ability to mimic. Prior to cell invasion, the adult mites stay for one day to several months (during the periods when the bee colony raises no brood) on adult bees. So cell-invading mites carry the mark of adult bees, e.g. substantial amounts of $C_{31:1}$ and $C_{33:1 \text{ and } 2}$ in *A. mellifera* colonies, and could be detected on a larva. This mimicry would also have no more protective value in the case of *A. cerana* if larvae of this species carry a HC profile different from the workers as seen in *A. mellifera*. *Varroa* walking speeds (excluding non moving periods) are slightly higher in controls in the membrane bioassay (2.85 mm s^{-1}) than on other substrates such as a servosphere (2.5 mm s^{-1}; chapter 4.1.) or a flat arena (1.97 to 2.24 mm s^{-1}; Colin et al., 1992). Speeds of 4 mm s^{-1} or higher recorded for *Varroa* on membranes treated with 5.9 leq of the active fraction are such that the width of the brood cell at its maximum of 5 to 7 mm would be covered in 1 to 2 seconds (chapter 4.2. and 4.4.7.). The observed increase in walking speed on increasing doses of cuticle extract may well serve to minimize the time of *Varroa* exposure to worker bees during cell invasion. This may represent an adaptation to defensive elimination behavior of *A. cerana* workers. Such a behavior to reduce the duration of a risk-prone situation may bridge the gap in the chemical camouflage. Occasional observations (unpublished) of mites on a larva moving without hesitation into the cleft between larval body and cell wall confirmed this hypothesis.

5.8. Synthesis of results

Summing up the results of this study gives the following picture:

⇨ *Varroa* can locate an adaequate odour source in using the wind direction.

⇨ The mites discriminate between olfaction and contact-chemoreception as seen by their responses to PA.

⇨ The mites discriminate between saturated and unsaturated HC's, and between staight-chain and branched alkanes.

⇨ The mites recognize the alkanes by chain-length (*n*-C21:0 was active, *n*-C23:0 inactive).

⇨ Synergism between HC's in elliciting behavioral responses in *Varroa* were observed. Mixtures of two singly-tested inactive *n*-alkanes, e.g. *n*-C23:0 and *n*-C25:0, were active. The low activity thresholds for TLC subfraction F1A containing the alkanes and that of the synthetic *n*-alkane mixture also suggest synergistic effects.

⇨ Bees, larvae and wax show some characteristics in the surface product profiles which may be candidates to serve *Varroa* as chemostimuli to discriminate between the hive components mentioned above (Table 11).

⇨ Circumstantial evidence exists to suggest that *Varroa* recognize the appropriate host life-stage. Host recognition and location within the bee hive is most probably controlled by locally correct concentrations of odours and contact-chemostimuli, either on their own or in combination with other cues, e.g. substrate quality.

***Table 11:** Hexane-soluble semiochemical content of wax, adult bees and larvae*

hive component	surface products
wax	PA [1,2], *n*-alkanes [1,2,3], few *br*-alkanes [1,2,3], HC's less than 20 % [1]
adult bees, older than 3 days	PA [1], *n*-alkanes [1,3,4], few *br*-alkanes [1,3,4], HC's more than 50 % [1]
adult bees, 0-1 day old	(PA ?), *n*-alkanes [3,4], much *n*-C23:0 [3,4], much *br*-alkanes [3,4]
larvae, 8-9 days old	PA [3], *n*-alkanes [3], few *n*-C23:0 [3], much *br*-alkanes [3], much FAE's [5]

[1] Blomquist et al., (1980)
[2] Tulloch (1980)
[3] this study
[4] Francis et al. (1989)
[5] FAE's = fatty acid esters; Trouiller et al. (1991)

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Curriculum vitae

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1988	first stay at the Department of Apiculture, Forschungsanstalt für Milchwirtschaft (FAM), Liebefeld / BE
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