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Odour-mediated transfer to brood cells of its honeybee host, *Apis mellifera*, and olfactory cell responses of *Varroa destructor* to volatiles

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Abstract

In a laboratory behavioural assay *Varroa* left nurse bees to enter brood cells with both drone and worker *Apis mellifera* larvae on combs*,* but not to empty cells on combs. *Varroa* colonised the brood through a 3-mm mesh plastic screen that prevented access by infested bees to the larvae. More infesting *Varroa* mites left the bees for drone brood than for worker brood cells. *Varroa* also transferred from nurse bees to combs without larvae but with food jelly. Electrophysiological recordings of action potentials from receptors in the olfactory sensilla on the first leg tarsal pit organ of *Varroa* show that the mite can sense bee-associated volatiles.

Introduction

The ectoparasitic mite *Varroa destructor* (Anderson and Trueman, 2000), originally infesting the Asian honeybee, *Apis cerana*, is currently causing severe damage to the European honeybee, *Apis mellifera*, worldwide (Oldroyd, 1999; Spivak, 1999). The female mite infests adult bees as well as larvae. To reproduce, the mite must transfer from adult nurse bees into the brood cells (Fuchs and Müller, 1988; lfantidis, 1988, 1991; Boot, 1995). The mite invades the brood cell just before operculation (Ifantidis, 1988; Boot, 1995; Berg et al., 1999). *Varroa* then hides behind the 5th instar larva until operculation by immersing itself in the proteinaceous food juice secreted from the hypopharyngeal glands of nurse bees (Fluri et al., 1982; Moritz and Crailsheim, 1987). It is assumed that the mite is guided by chemical cues while infesting the brood cell (Le Conte et al.,1989; Rickli et al., 1992). Trophallaxis plays a central role in information transfer in the beehive (Crailsheim, 1998, Camazine et al., 1998) through exchange of food jelly as well as recognition cues (Breed 1998). Nurse bees – the preferred hosts of phoretic mites – are specialists in metabolising pollen to food juice. There is a possibility that *Varroa* is hitchhiking on this information channel of the insect society. In a contact bioassay Calderone and Lin (2000) detected a repellent effect of royal jelly on *Varroa* but no arrestment or repellent effect of worker- or drone jelly. Nazzi et al. (2001), however, showed a preference for worker- and drone jelly over the control in a laboratory choice test.

If indeed chemical stimuli guide *Varroa* to a resource, it can be argued that the most sensitive detector for biologically active substances is the mite itself through adaptively “matched filters” of its sensory system (Wehner, 1987). Action potentials recorded from receptor cells in olfactory sensilla tell whether volatile products can be detected by the mite’s sensory system as in other arachnid and insect ectoparasites (Steullet and Guerin, 1992; Guerin et al., 2000). Substances perceived by the mites can then be tested as cues for odour–mediated behaviours in a bioassay. *Varroa* uses its front legs in the same way as insects use their antennae (Rickli et al., 1992, 1994); they are only rarely used for movement and are more frequently displayed in the air. Olfactory sensilla occur on the front legs of *Varroa* (Milani and Nanelli, 1989; Ramm and Böckeler, 1989) as in other arachnids (Hess and Vlimant, 1982) where the most distal tarsal segment bears a pit organ with sensory hairs in it. The ultra-structure and histology of these sensilla in *Varroa* bear a striking similarity to olfactory sensilla of other arthropods, so it can be assumed that at least some of these sensilla house receptors involved in the perception of volatile host cues.

Here we show that volatiles from worker and drone brood are relevant for brood cell invasion by *Varroa* in a behavioural assay where mites move from nurse bees to honeybee brood cells in the absence of contact by the nurse bees with the brood. Secondly, we show with electrophysiological recordings that *Varroa destructor* can perceive bee-associated volatiles by means of receptors in wall-pore sensory hairs of the tarsal pit organ.

Materials and methods

Behavioural assays

A simple behavioural assay was developed to measure transfer of *Varroa* mites from nurse bees to the honeybee brood cells for reproduction. For good reproducibility the test had to be kept as simple as possible, but also had to include a minimum of natural hive conditions. The “donor bees” infested with *Varroa* were collected from brood frames of test–hives (Dadant hive system). These colonies were not treated against *Varroa* in the previous season to permit a high mite infestation. The bees for tests were picked from the frame manually with fine latex gloves to prevent handling contamination. While holding the bees by their wings, they are checked for *Varroa*. The number of *Varroa* per bee and the parasite’s position on a bee was recorded. The infested bees were immediately transferred into one compartment of a small transparent polystyrene test box 5x5x5 cm (Fig. 1; Semadeni AG, Switzerland). Test boxes were divided in two by a plastic 3-mm mesh screen. Pieces of 5X5 cm brood comb with either 100 worker cells or 75 drone cells on one side were cut out of brood nests. This “acceptor brood” originated from almost *Varroa*-free hives. These

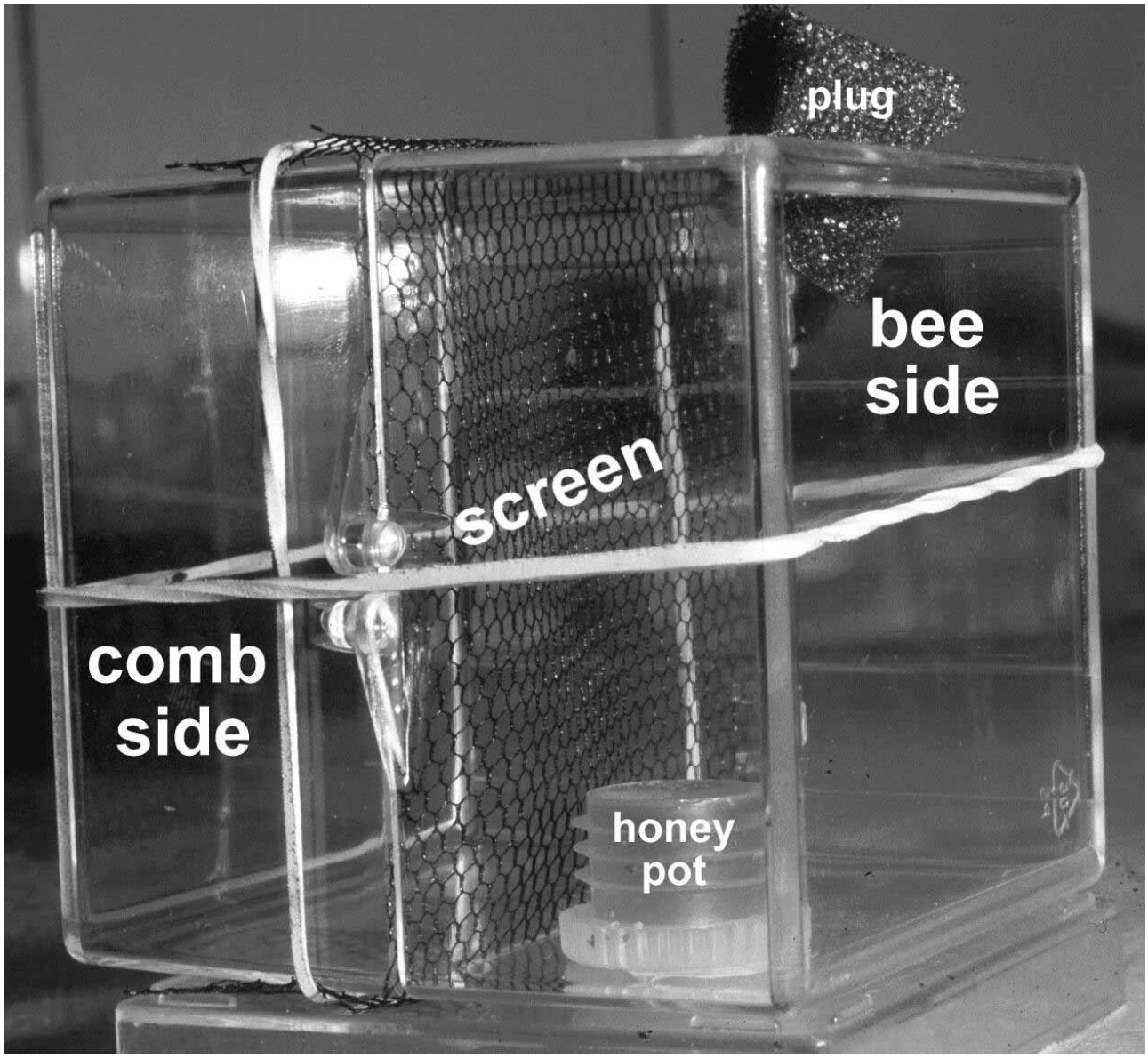


Fig. 1: A small transparent polystyrene test box 5x5x5 cm, divided into comb and bee sides by a 3-mm mesh plastic screen, with a plastic ca. 1 ml honey pot on the floor of the box and a ∅ 6 mm hole with a plug of foam rubber for the introduction of the bees and air exchange at the top. The screen is fixed and the two sides of the box are held together by rubber bands.

brood combs were inserted, at their original orientation in the hive, in the other compartment of the test boxes. The screen prevented the nurse bees from direct contact with the larvae, except for some outstretched 5th instar larvae that had already started to spin the cocoon. Although the screen touched the comb surface, no operculation took place even though some larvae spun their cocoons against the screen. A comparison between boxes with and without a plastic screen tested the influence of *Varroa* transfer to brood from nurse bees that were prevented or not from access to the larvae. Boxes without the screen had to be turned 90° from the normal vertical comb position as the larvae otherwise tended to leave the brood cells early in the test period. To test the influence of food jelly all larvae in the "acceptor comb" piece were carefully removed from the brood cells with fine forceps. This took place just before the test started or the larva-free comb with only jelly remaining in the brood cells was deep-frozen for later use.

Preliminary tests served to establish an adequate test duration, climatic conditions, and improve the bee density inside the test boxes. The number of bees per test box was increased from 25 in the first tests to 50 in the following series; fifty bees correspond to ca. one layer of bees on the 5x5cm comb. Each test box contained 50 bees, 25 bees infested with *Varroa* plus 25 non-infested bees. The brood comb was as homogenous as possible both in terms of brood cell distribution and age of the brood (5th instar larvae near operculation), although this could not be perfectly reproduced to the same degree in all test series. The distribution of the cell types on the piece of comb was sketched before and after a test and the following were noted: empty cells, honey cells, pollen cells, cells with eggs, dead larvae, small larvae (1st to 4th instar larvae not yet filling the bottom of the cell completely), big larvae (5th instar larvae filling out the bottom of the cell completely), outstretched larvae (5th instar larvae, no longer coiled at the bottom of the cell), and operculated cells.

The closed test boxes were put in an incubator at 32°C, 60-70% RH; T°C and RH was monitored in the incubator as well as inside a control test box with 50 bees and a piece of brood comb (Ecolog, Elpro-Buchs Ag, CH-9471 Buchs, Switzerland). After 24h the animals in the test boxes were anaesthetised with 100% CO_2 and deep-frozen. A test duration of one day was a good compromise in order to provide *Varroa* enough time to enter the brood cells and to avoid larval starvation. The number and distribution of *Varroa* mites in the two parts of the test boxes, on the bees and in the comb cells was determined under a stereomicroscope. After this inspection, comb pieces were rinsed with hot water to check for any mites that escaped detection. In this manner between 82 and 100% of the introduced *Varroa* were accounted for. Three categories of *Varroa* were distinguished: *Varroa* on the bee side of the separating screen, *Varroa* on the comb side of the screen and a subdivision of the latter, i.e. *Varroa* inside larval cells. Numbers were expressed as the proportion of the number of *Varroa* counted after the test and analysed under two criteria: 1) the proportion of *Varroa* found on the comb side of the test box as a measure of mites that left the nurse bee, and 2) the more strict criterion of the subgroup of comb-side *Varroa* found inside brood cells, normally hiding behind the larvae in the food jelly.

Microscopy

Mites were prepared for examination by scanning electron microscopy (SEM, Philips XL20 at 25 kV) by ultra-sound cleaning, fixed and extracted in a 1:1 mixture of ether-chloroform at room temperature in a Soxhlet apparatus for several days before being dehydrated. They were then critical point dried in CO_2 in a Baltec CPDO30. The mounted specimens were gold sputtered under argon in a Baltec 9CD005 apparatus (Hess and Vlimant, 1982).

Electrophysiology

Ten to 20 bees were picked manually from a brood-frame, checked for *Varroa* infestation and the infested ones transferred to a wooden 10x8x3.5 cm Liebefelder bee-holding box. Infested bees with a supply of food were kept at 28°C and 70-80% RH for up to 14 days. For electrophysiology, the mite was removed from the bee with a pointed moistened paintbrush. The living animal was glued with sticky tape over the scutum on the front side of a Plexiglass® piece in the shape of a house (approx. 1x1x1 cm). The front legs of the mite were fixed in wax under a stereomicroscope onto the "roof" of the Plexiglass® piece with the pit organs free. The reference microelectrode either

of tungsten or a drawn-out borsilicate glass electrode was inserted by means of micromanipulator into a leg. The recording electrode (tungsten or glass) was likewise inserted at the base of one of the sensilla in the pit organ of a front leg. Either recording electrode type had to make contact with the sensiliar lymph surrounding the receptor cell dendrites within the sensilla via wall-pores (Guerin et al., 2000). The whole set-up was bathed in a charcoal-filtered and moistened airflow (90% RH, 1.4 m/s; Steullet and Guerin, 1994). Test stimuli were injected into this airflow at 1 ml/s from a plastic stimulus syringe (5ml) operated by an electronically controlled solenoid valve. The valve switched to the test stimulus system for 1.6 or 3 s from a continuous control of identical flow rate and configuration. Test stimuli were living bees or single synthetic volatiles. For each test chemical a 10 µl solution was placed on a filter paper strip and, after evaporation of the solvent, the strip was introduced into the stimulus syringe. An oscilloscope and a loudspeaker monitored action potentials (spikes) produced by the olfactory receptor cells in the olfactory sensilla in response to an adequate stimulus. The electrophysiological signal was simultaneously recorded via a multi-channel-data-recording-Box connected with the USB-port or a data acquisition board (Syntech, NL) in a personal computer and stored for later analysis by a spike analysis software (Autospike32, Syntech, NL).

Results

Behavioural assays

There was no significant difference in the number of *Varroa* entering brood cells in test boxes with the separating screen between the brood comb and the nurse bees and in test boxes without such a separation (Fig. 2); no access by the nurse bee to the brood cells was needed for the mites to leave the bees. In our parallel tests a significantly higher proportion of mites transferred from bees to drone brood cells than to worker brood (Fig. 3). However, the number of *Varroa* found inside the brood-cells was only significantly different between drone and worker cells in the May test series

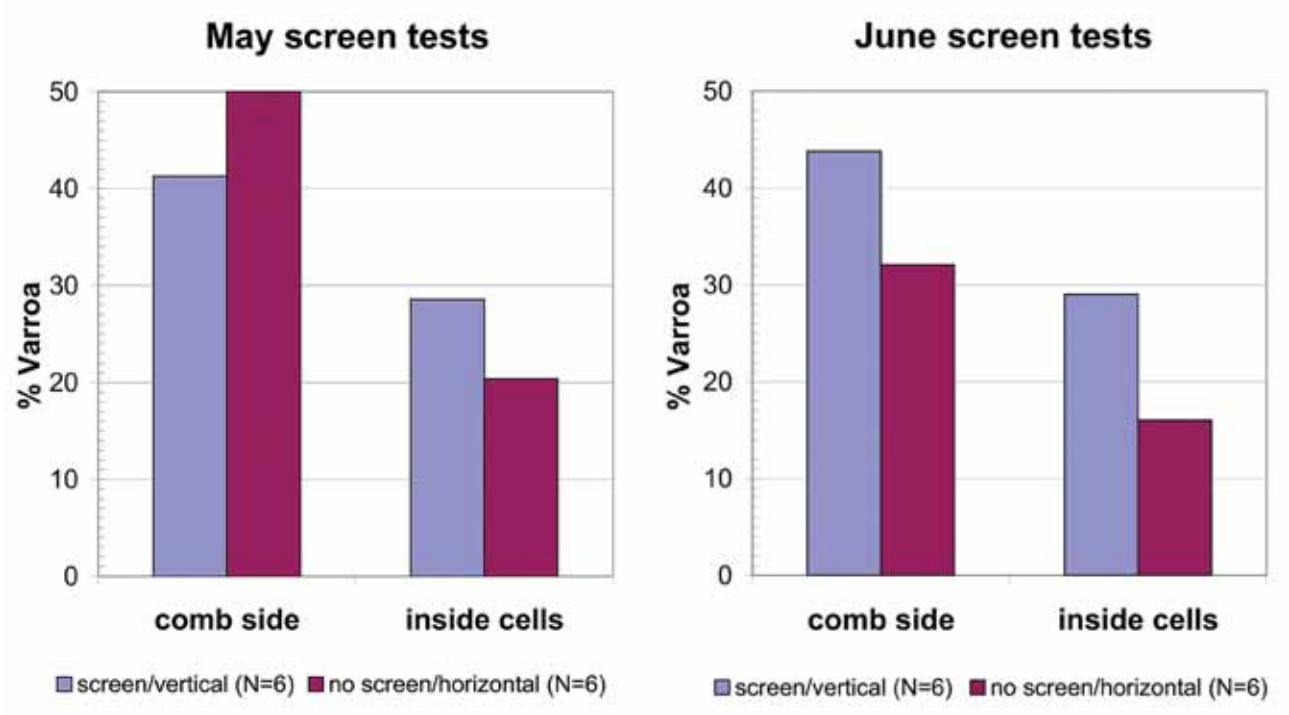


Fig. 2: Percentage of *Varroa* leaving bees in test boxes where nurse bees had access or none to brood cells. Numbers of *Varroa* on the comb side and numbers inside the brood cells were the same in both types of boxes after 24 h: *Varroa* on comb side of the box (May tests: N=6, t-test, P=0.89; June test: N=6, t-test, P=0.32) and *Varroa* that entered the cells (May tests: N=6, t-test, P=0.31; June test: N=6, t-test, P=0.15). The proportion of *Varroa* on the comb side of the boxes was the same for the May tests and the June tests (27 *Varroa* of 54 in 6 boxes with no screen and 26 *Varroa* of 63 in 6 boxes with the screen in May; 42 *Varroa* of 131 in 6 boxes with no screen and 60 *Varroa* of 137 in 6 boxes with the screen in June).

due to the lower proportion of *Varroa* leaving bees in June. The high variation in the proportion of *Varroa* leaving the bees between the test series was not correlated with the number of non 5th instar larva cells in the comb piece, i. e. empty cells, honey cells, pollen cells, cells with eggs, dead larvae and small larvae; 5th instar larva cells were always in the majority.

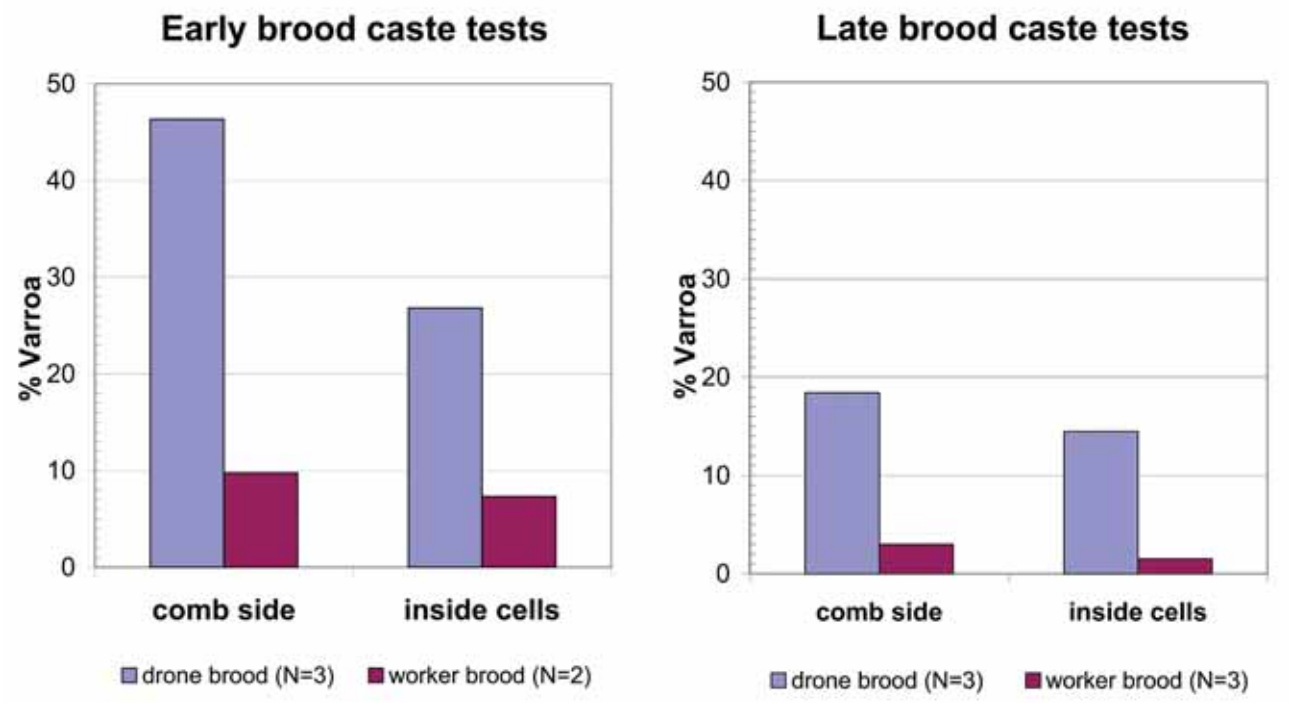


Fig. 3: Percentage of *Varroa* leaving bees in test boxes with drone brood and with worker brood. In both test series (early June tests with 5 test boxes, i.e. 2 with worker and 3 with drone brood, and late June tests with 6 test boxes, i.e. 3 with worker and 3 with drone brood) a significantly higher proportion of *Varroa* were counted on the comb side with drone brood than with worker brood (two sided t-test, $P < 0.01$ in both tests). With the stricter criterion of the proportion of *Varroa* inside the brood cells, the difference was only significant in early the June tests (two sided t-test $P < 0.01$), and not in late June (two sided t-test $P = 0.10$). The overall proportion of *Varroa* leaving hosts in late June was 11% compared to 34% in early June (38 *Varroa* of 82 were found on drone brood in 3 boxes and 4 *Varroa* of 42 on worker brood in 2 boxes in early June, and 14 *Varroa* of 76 on drone brood in 3 boxes and 2 *Varroa* of 67 on worker brood in 3 boxes in late June)

In several control series in 1999 and 2001 where empty cells were compared to worker brood cells, a significantly higher number of *Varroa* transferred to the comb side where worker brood was present (Fig. 4). The high proportion of mites still on bees in tests with empty clean drone or worker cells (newly built drone comb or empty worker brood comb from the previous year) proved that the test situation in itself was not responsible for the mites to transfer to the comb side.

The proportion of *Varroa* transferring to combs with worker jelly alone in the brood cells (brood comb with mostly 5th instar worker larvae removed) was significantly higher than the proportion transferring to combs with clean empty cells (comb material not previously involved in a brood cycle; Fig. 5) but significantly lower than the proportion of *Varroa* transferring to combs with living 5th instar worker larvae.

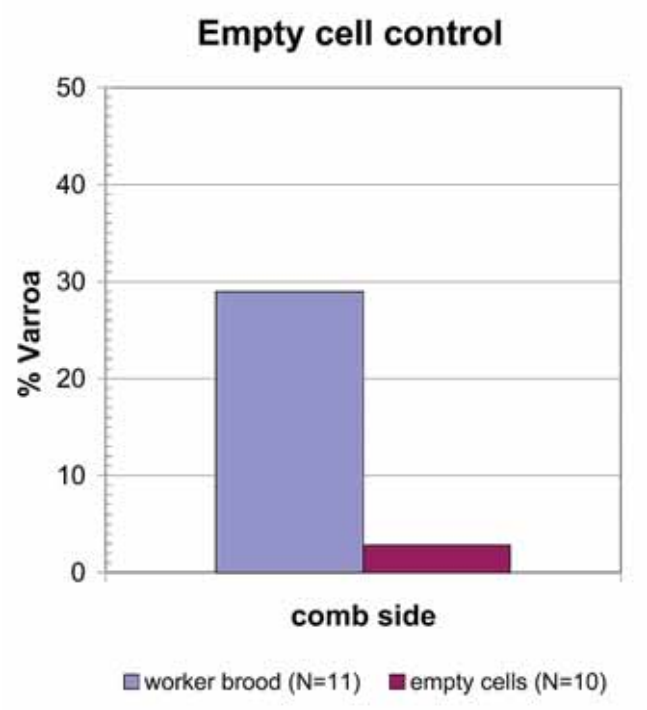


Fig. 4: Percentage of *Varroa* found on the comb side in test boxes with worker brood or empty cells (pooled data from four test series in 1999 and 2001, 11 boxes with worker brood and 10 boxes with empty cells); *Varroa* numbers on the comb side was significant higher in the boxes with worker brood (99 *Varroa* of 325 in 11 boxes with worker brood and 8 *Varroa* of 276 in 10 boxes with empty cells, t-test significant P<0.001).

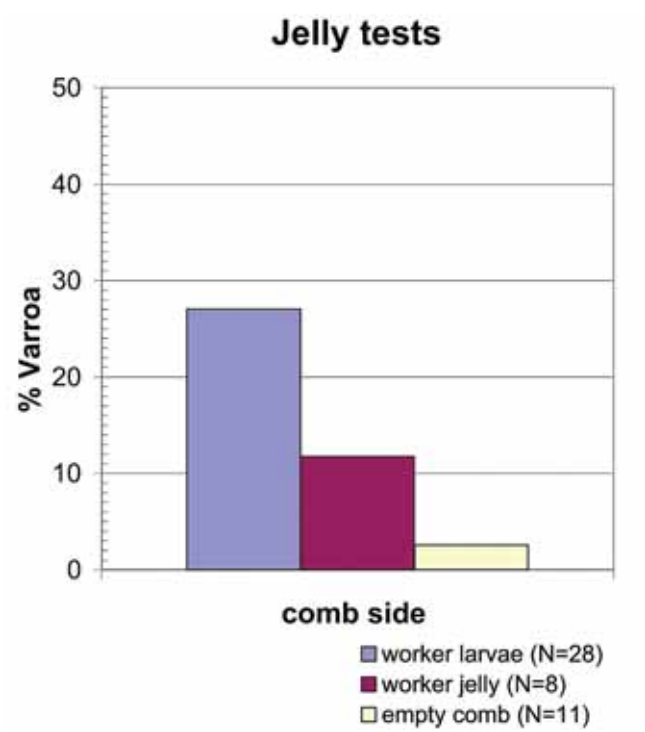
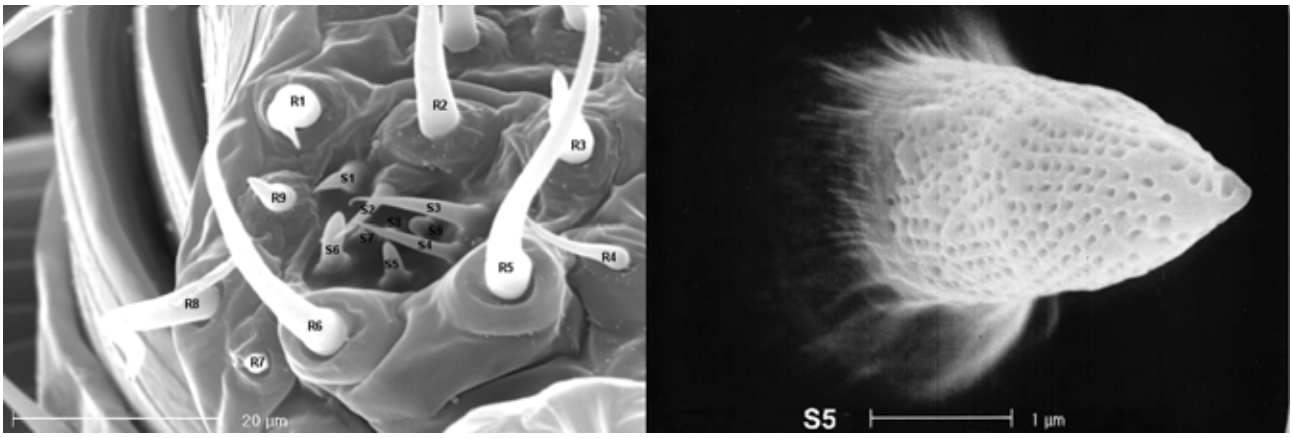


Fig. 5: Percentage of *Varroa* on the brood side of test boxes with worker jelly (brood comb with worker larvae removed) compared to worker brood and empty cells (pooled data from nine test series in 1999 and 2001, 28 boxes with worker brood, 8 boxes with worker jelly alone and 10 boxes with clean empty cells). There was a significant difference between *Varroa* numbers transferring to worker larvae and to cells with jelly (two sided t-test, $P < 0.002$) as between cells with jelly and clean empty cells (two sided t-test, $P < 0.017$).

Electrophysiology

An increase in of action potential frequency was recorded with glass-electrodes at the base of olfactory wall-pore sensilla in the pit organ of *Varroa* (Fig. 6) in response to stimulation with the odour of living bees .The synthetic volatiles benzaldehyde, salicylaldehyde and methylsalicylate (Fig. 7) provoked an increase in spike frequency in wall-pore sensilla. The solvent control had no



a) **b)**

Fig. 6: Electron micrograph of the pit organ on the front leg tarsus of *Varroa destructor* with sensilla (S1-S9) inside the pit and longer sensilla (R1-R9) surrounding it **a)**. In the centre of the pit there are wall-pore olfactory sensilla S1, S3, S4 and S5 (S5 in the second micrograph, **b)**.

influence on the firing rate of olfactory receptors.

a) benzaldehyde 100mg

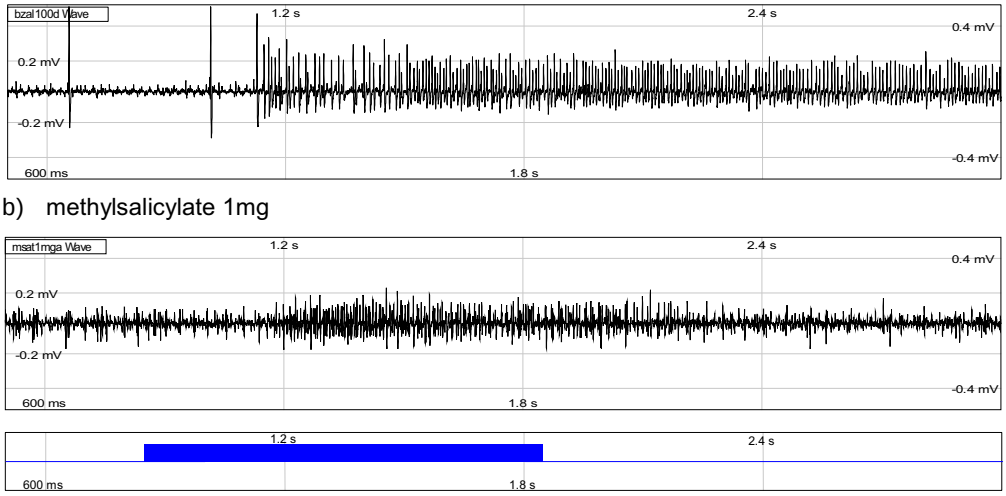


Fig. 7: Action potentials recorded from olfactory sensilla in the pit organ of *Varroa destructor* with tungsten microelectrodes after stimulation with benzaldehyde (100 mg stimulus source) **a)** and methylsalicylate (1 mg stimulus source) **b)**; the bar indicates the duration (1.6 s) of the stimulation.

Discussion

The ability of brood cells with living larvae or food jelly but not clean empty cells to enhance the probability of *Varroa* be found on the comb side of test boxes demonstrates that cues associated with brood or food jelly are involved in the decision by female mites to leave host bees or to stay on the comb side. *Varroa* can discriminate between olfactory cues in an olfactometer (Le Conte et al., 1989) and on a servosphere (Rickli et al., 1992; Rickli, 1994). In the tests where either 5th instar

worker or drone larvae were presented in parallel tests, *Varroa* transferred to brood more often when drone larvae were available. This implies a higher motivation to leave bees in the proximity of drone larvae than of worker larvae or to stay longer on the drone comb side after having left the bees. Several authors have reported preferences by *Varroa* for drone cells compared to worker cells in the beehive (Otten and Fuchs, 1988; Le Conte et al., 1989; Fuchs, 1990; Boot et al., 1992, 1994; Vandame, 1996). Drone comb pruning and *Varroa* trapping in drone combs by beekeepers serves as a method in alternative *Varroa* control concepts (Charrière, et al., 1999; Calis et al., 1999). Whether mites can discriminate between drone and worker brood cells in a natural environment had not yet been shown (Beetsma et al., 1999), but laboratory tests and our results with “minimal hive conditions” show that it is highly probable. In drone cells there is a threefold longer time window for cell invasion (14 to 20h in worker cells and 40-50h in drone cells; Boot et al., 1992; Beetsma et al., 1999). This mechanism cannot be responsible for the higher infestation rate of drone cells in tests with a fixed duration of 24h. The test duration is close to the time window for cell invasion in worker brood, which is probably a bit prolonged in our test situation, where no operculation takes place. There is also evidence that the raised borders of drone cell patches contribute to the clumped distribution of infested brood cells on mixed worker/drone brood combs (Kuenen and Calderone, 2000). In our parallel tests with either worker or drone comb such borders didn’t exist. Distance from the cell rim to the larva has found to be an important factor for the cell invasion behaviour of *Varroa* (Goetz and Koeniger,1993). They found that cells are more attractive to *Varroa*, when they have been shortened by filling the cell bottom with wax. This can be interpreted as a closer distance for the searching *Varroa* to the source of volatile compounds in the brood cell. Food jelly is a possible candidate as a source of such chemical cues, even if the involvement of other components of brood cells or cues leftover from the removed larvae (i.e. cuticular hydrocarbons and methyl esters, Rosenkranz and Aumeier, personal communication; aliphatic alcohol’s and aldehydes, Donzé et al.1997) cannot yet be excluded. It is likely that volatile olfactory cues were involved in the transfer to brood. In this study nurse bees harbouring *Varroa* did not have contact with the brood cells. We have shown here that brood cells with jelly causes *Varroa* to transfer more frequently to the comb compared to clean empty cells. Also that jelly is found in much higher quantities in drone cells compared with worker cells supports the jelly hypothesis. The trophallaxis, the exchange and distribution of jelly to the foragers, the queen and the larvae plays a key role in the organisation and communication in the honey bee society (Crailsheim, 1998, Camazine et al., 1998). Nurse bees, the preferred hosts of phorectic mites, are exclusively responsible for metabolising pollen into jelly, the only protein source for her hive members. There is a correlation between the seasonal reproduction phase of *Varroa* and the seasonal peak of hypopharyngeal gland development and blood protein contents in nurse bees (Fluri, 1982; Hrassnigg and Crailsheim, 1998).

Varroa destructor is equipped with olfactory wall-pore sensilla in the tarsal pit organ on the dorsal face of each front leg (Milani and Nanelli, 1989; Ramm and Böckeler, 1989) and our electrophysiology experiments show that the mite is able to perceive volatiles from in air. The small dimension of the olfactory pit organ makes it very difficult to get good recordings from the sensory cells in the olfactory sensilla (Endris and Baker, 1993). Among the synthetic compounds that provoked olfactory responses, benzaldehyde is known as a volatile in royal jelly and adult drones (unpublished data from Swiss Bee Research Centre, Swiss Federal Dairy Research Station, Berne) and benzaldehyde and methylsalicylate are also constituents of pollen, flowers and honey (Maga, 1983). Receptors for these products are known from arachnids. A benzaldehyde receptor is described in sensilla olfactory on the tarsus of the tick *Amblyomma variegatum* (Steullet and Guerin, 1994b) and a methylsalicylate receptor occurs on the mite *Phytoseiulus persimilis* (de Bruyne et. al., 1991). Brood cell invasion behaviour is probably influenced by a multiple of cues detected by different sensory pathways in *Varroa destructor*. This leads to an average ten fold higher infestation rate of drone brood than worker brood of *Apis mellifera* (Martin, 1998) and its precise timing in the beehive.

Zusammenfassung – Flüchtige Geruchsstoffe beeinflussen bei *Varroa destructor* den Abstieg von den Ammenbienen auf die Brut ihres Wirtes, *Apis mellifera*, und führen zu Antworten der olfaktorischen Rezeptorzellen.

Die Milbe *Varroa destructor* muss, um sich im Bienenvolk zu reproduzieren, von den Ammenbienen in die Zellen der verdeckelungsbereiten Brut absteigen. Dort kann sie sich durch Eintauchen in den proteinreichen Futtersaft verbergen. Es wird angenommen, dass sie sich dabei auch durch Geruchsignale leiten lässt. Ein Labortest zum Absteigeverhalten soll zeigen, ob von den Brutzellen ausgehende Signale die *Varroa*milben zum Wechsel von den Ammenbienen auf die Brut bewegen können. Dazu wurden in ein Plexiglaskästchen von 5x5x5 cm (Fig. 1) in der einen Hälfte 25 von *Varroa* befallene und 25 varroafreie Bienen aus einem *Varroa*-Volk eingesetzt und in die andere Hälfte ein varroafreies Wabenstück eingefügt. Nach 24 h im Brutschrank bei 32°C wurden die Tiere in den Kästchen mit CO2 betäubt und anschliessend tiefgefroren, um den Aufenthaltsort der Milben für die Auswertung festzuhalten. Als Mass für den Abstieg der Milben von den Trägerbienen diente derjenige Anteil der *Varroa*, der sich bei Versuchsende in der Wabenhälfte des Testkästchens oder – als zweites schärferes Kriterium – diejenige Untergruppe der Milben, die sich in einer Larvenzelle befand. Ein Kunststoffgitter mit 3 mm Maschenweite zwischen den Bienen und der Wabe mit der verdeckelungsbereiten Brut hatte keinen Einfluss auf die Absteigerate (Fig. 2). In den Kästchen mit Trenngitter war die Absteigerate signifikant höher, wenn eine Wabe mit Drohnenbrut, anstatt einer Wabe mit Arbeiterinnenbrut, angeboten wurde (Fig. 3). Ein Stück leere ausgebaute Wabe konnte die *Varroa* nicht zum Verlassen der Bienen bewegen (Fig. 4), hingegen führten zuvor bebrütete Waben mit nur Futtersaft (Zellen mit frisch ausgezupften Arbeiterinnenlarven) zu einer Absteigerate zwischen derjenigen von Kästchen mit lebenden Arbeiterinnenlarven und Kästchen mit leeren Zellen (Fig. 5). Futtersaft spielt eine zentrale Rolle beim Futter- und Informationsaustausch im Bienenvolk, der Trophallaxe. *Varroa* könnte von diesem Informationskanal profitieren. Rasterelektronische Aufnahmen zeigten, dass *Varroa* auf der Oberseite der Vorderbeine ein Grubensinnesorgan besitzt. In dieser Grube befinden sich Wandporen-Sensillen, die sich im Vergleich mit von anderen Arachniden und Insekten beschriebenen Sensillen, als Geruchssinnesorgane deuten lassen (Fig. 6). Es gelangen uns elektrophysiologische Ableitungen von Aktionspotentialen an diesen Sensillen in Reaktion auf eine Reizung mit volatilen Geruchsstoffen (Fig. 7). Obwohl die Bienen durch das Trenngitter keinen Zugang zur Brut hatten, lösten Faktoren aus den Brutzellen das Absteigeverhalten der Milben aus. *Varroa* besitzt auf den Vorderbeinen Geruchsinnesorgane und kann, wie die elektrophysiologischen Ableitungen gezeigt haben, flüchtige Geruchsstoffe aus der Brut oder von Bienen wahrnehmen und darauf reagieren. Die Verhaltensversuche zeigten, das Faktoren aus der Brut oder dem Futtersaft beim Auslösen des Absteigeverhaltens der Milben von den Ammenbienen oder der Entscheidung, auf den Waben zu bleiben, mitbeteiligt sind.

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