

Review of the orientation behaviour in the bee parasitic mite *Varroa destructor*: Sensory equipment and cell invasion behaviour

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Review of the orientation behaviour in the bee parasitic mite *Varroa destructor*: Sensory equipment and cell invasion behaviour. - Cell invasion behaviour is a crucial phase in *Varroa destructor* (Anderson & Trueman, 2000) life history. This review gives a survey of what is known about the sensory equipment of *Varroa* suited for its orientation. Most attractive for *Varroa* females are nurse bees and drone larvae just before the capping by the nurse bees. In search of cues to determine the sex and age of the brood, the whole spectrum of approaches from direct observation of the invasion behaviour in observation hives to electrophysiological recordings of the sensilla was used. There was no single key factor identified to be responsible for the host recognition, but rather a complex mixture of chemical compounds from the brood and physical parameters of the comb. In consideration of our limited understanding of host parasite interaction there is no solution in sight for a biological *Varroa* treatment regime based on interference with *Varroa* orientation during the brood invasion phase.

Keywords: *Varroa destructor* - *Apis mellifera* - olfaction - host finding - orientation..

INTRODUCTION

The ectoparasitic mite *Varroa destructor* (Anderson & Trueman, 2000) originally infested the Asian honey bee, *Apis cerana*, and is currently causing severe damage to the European honey bee, *Apis mellifera*, worldwide (Spivak, 1999). Due to the rapid expansion of its range from East Asia all over the world, with the only exception of Australia, in the last twenty years, *Varroa* has become the most serious problem in beekeeping. Together with the applied research on pest management and the development of treatment concepts (Imdorf *et al.*, 2003), there have also been twenty years of basic research on a better understanding of what is going on in our beehives regarding this introduced bee parasite.

The female mite infests adult bees as well as larvae. To reproduce, the mite must move from adult nurse bees into the brood cells (Fuchs & Müller, 1988; Ifantidis, 1988, 1991). The mite invades the brood cell just before operculation (Berg *et al.*, 1999; Boot, 1995; Ifantidis, 1988). *Varroa* then hides behind the 5th instar bee larva until operculation by immersing itself in the food juice (Donzé & Guerin, 1997).

We will try to shed light on research done on the mites' orientation behaviour in the context of the complex host-parasite interaction. For a better understanding of this behaviour we will take a short look at what is known about the sensory morphology, neurobiology and sensory ecology of *Varroa*. The aim of this review is to bring together the current knowledge about the different possible orientation mechanisms involved in the host finding behaviour of *Varroa destructor*, with a special focus on its olfactory orientation.

SENSORY EQUIPMENT OF VARROA MITES

Which are the sensory modes that help the bee parasitic mite *Varroa destructor* to orient itself in the bee colony? The knowledge on the sensory equipment of mites in general and of *Varroa destructor* in particular is still relatively scarce. Almost the whole body of *Varroa*, including the eight legs and the mouthpart with its appendages, is covered with different types of hairs (Fig. 1). At least part of them has mechanoreceptive functions.

PERCEPTION OF CHEMICAL CUES: *Varroa* uses its two front legs in the same way as insects use their antennae (Rickli *et al.*, 1992). These legs are only rarely used for movement and are more frequently displayed in the air. Scanning electron microscopy (SEM) shows the presence of a sensory pit organ (Fig. 2) on the front leg tarsi of *Varroa destructor* (Milani & Nannelli, 1988; Ramm & Böckeler, 1989). The pit organ consists of nine sensilla (S1-S9) inside the pit and nine longer hair sensilla (R1-R9) surrounding it. The structure reminds one of Haller's organ found in ticks (Hess & Vlimant, 1982, 1983a, b; Sonenshine *et al.*, 1986). According to Ramm and Böckeler (1989) the sensilla S1 and S3 to S5 are classified as wall pore sensilla. 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 some of these sensilla house receptors, which are involved in the perception of volatiles. Sensilla S7, S8 and S9 are non-pore sensilla serving as hygro- and thermo-receptors, whereas the morphology of sensilla S2 and S6 indicates a gustatory role. The edge bristles surrounding the tarsal pit are also divided in two groups. R1 to R3, R5, R6 show the characteristics of a contact chemo-receptor as seen in S2 and S6, while R4, R7, R9 have characteristics that remind us of sensilla seen in other arthropods serving as chemo-receptors with additional thermoreceptive function.

Furthermore Liu & Peng (1990) described chemoreceptive sensilla on the palptarsus for *Varroa*. The large setae on the palptarsus of *Varroa* (Fig. 1) resemble sensilla chaetica of insects, thick walled sensilla with an apical pore. They are of the same type as the S2 and S6 sensilla in the tarsal pit organ, known as gustatory sensilla. The smaller setae on the palptarsus resemble sensilla trichodea of insects that may function in olfaction (Kaissling, 1971). Nuzzaci and collaborators (1992) described the presence of some specialised sensilla on the mouthparts, suspected by ultrastructural evidence to be gustatory chemical sensilla and mechanosensilla. These are probably related to the feeding behaviour of *Varroa* and not so much involved in orientation.

The most important sensual modes of *Varroa* are supposed to be the chemical senses, olfaction and taste. Therefore a great part of the research done on *Varroa* orientation focuses on this subject.

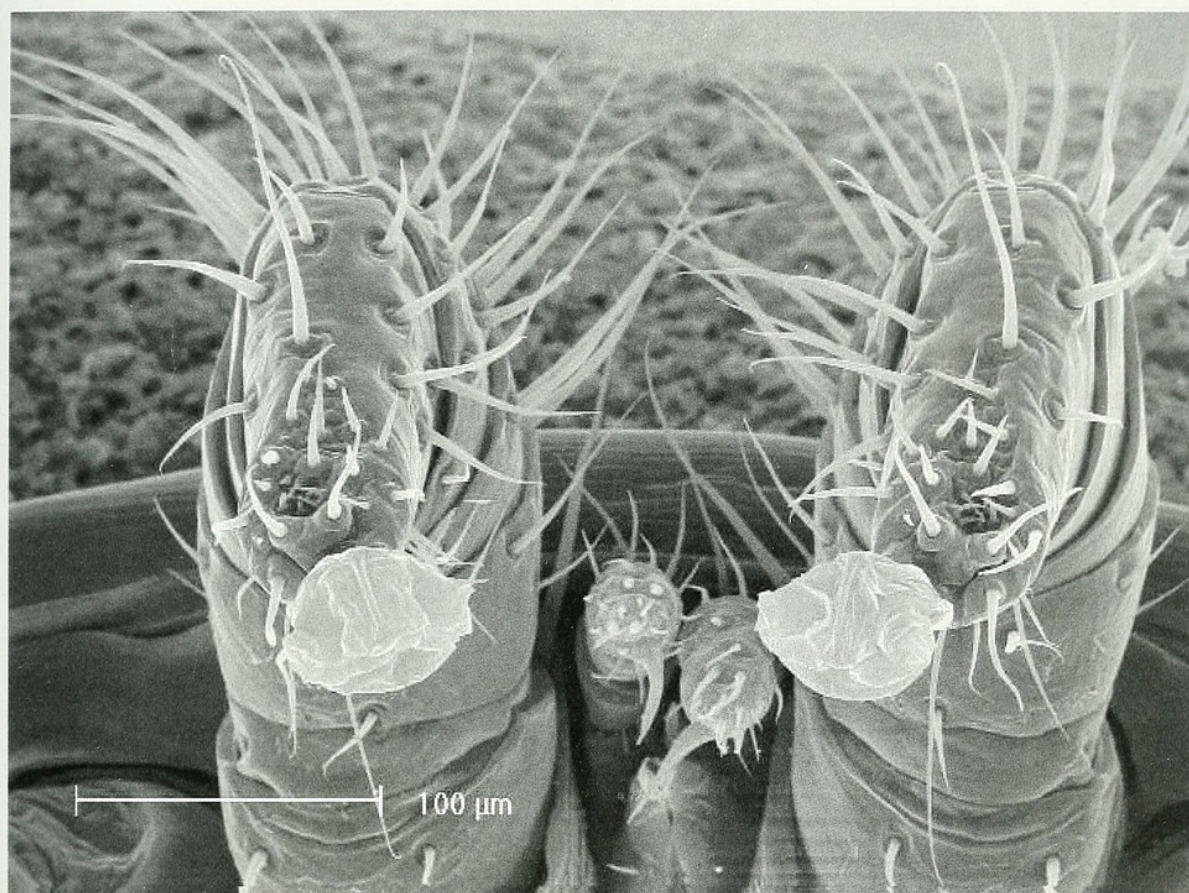


FIG. 1

Scanning electron micrograph of the front legs and the mouthparts, including the palptarsi in the centre, of *Varroa destructor*, covered with mechanosensitive and chemoreceptive hair sensilla (SEM by M. Vlimant, University of Neuchâtel).

The electrophysiology approach proved that *Varroa destructor* is indeed able to perceive volatile components in the air. Action potentials recorded from receptor cells in olfactory sensilla tell whether volatile products can be detected by the mite's sensory system. The synthetic volatiles, benzaldehyde, salicylaldehyde and methylsalicylate, provoked an increase in the firing rate of olfactory receptor cells (Blumer-Meyre *et al.*, 1998; Dillier *et al.*, 2001; Endris & Baker, 1993). Benzaldehyde is known as a volatile in royal jelly and on adult drones (Dillier *et al.*, 2002a), and benzaldehyde and methylsalicylate are also constituents of pollen, flowers and honey (Maga, 1983). Receptors for these products are known from some arachnids. A methylsalicylate and a benzaldehyde receptor were described from sensilla on the tarsus of the tick *Amblyomma variegatum* (Steullet & Guerin, 1994) and a methylsalicylate receptor occurs on the mite *Phytoseiulus persimilis* (de Bruyne *et al.*, 1991).

Varroa is well equipped with a machinery to detect a broad range of chemicals. Even if the mite's sensory system consists, in contrast to the situation in insects (Kaissling, 1971), only of a small number of specialised sensilla with a limited number of sensory cells, there is no evidence for a limitation to a single chemical cue used to invade brood cells. Each of its sensilla has individually different sensory characteristic and the neuronal network behind the sensilla is more important than the sheer number

of receptor cells (Wehner, 1989; Wehner, 1991; Wehner, 2003) in the task of cue detection in a complex chemical environment.

TEMPERATURE AND HUMIDITY PERCEPTION: It is known that mites are very sensitive to small differences in temperature and relative humidity (Hess & Loftus, 1984). Le Conte & Arnold (1987, 1988a, b), using a four-arm airflow olfactometer, found that *Varroa* is strongly attracted to the source with the higher air temperature, and to a lesser degree also by vibrations as produced by caged bees in the test. The mite can discriminate temperature differences of 1.2°.

Varroa shows a positive thermotaxis in laboratory bioassays. In a temperature gradient female mites prefer temperatures in the range of 26 to 31°C (Anchakova, 1977; Bairak, 1976; Rosenkranz, 1988), which is significantly below the normal temperature of about 35°C in a honey bee brood nest (Kraus *et al.*, 1998). However experiments of le Conte & Arnold (1988a) in which *Varroa* mites could choose to settle at a point on a temperature gradient showed that they selected a slightly higher mean temperature of $32.6 \pm 2.9^\circ\text{C}$. There also seems to exist a seasonal variation in the preferred temperature between summer- and winter-*Varroa* (31.8 to 37.3°C and 33.6 to 36.6°C, respectively) (Pätzold & Ritter, 1989). Nevertheless it should be kept in mind that the simultaneous control of humidity in a temperature gradient is difficult (a 1 °C increase in temperature results in a ~5% decrease of the relative humidity). The native host of *Varroa destructor* is *Apis cerana* and there it reproduces almost exclusively in drone brood (Anderson & Trueman, 2000; Boecking *et al.*, 1993; Boot *et al.*, 1997, 1999; de Jong, 1988; Oldroyd, 1999; Rath, 1992, 1999). The range of drone brood temperature of *Apis cerana* (30 to 34°C) is 0.4°C lower than the temperature range in *Apis mellifera* drone brood cells (Kraus *et al.*, 1998). This difference is very small compared to the uncertainties in measuring temperature preferences in *Varroa*. It does not explain the differences in *Varroa* behaviour in invading different host species. The observed, so called "preferences" of the mite in a temperature gradient probably reflect preferences for different relative humidities, or a combination of the two (Bruce *et al.*, 1997). This may explain why in most experiments the *Varroa* mite systematically seemed to prefer much lower temperatures than those observed in the brood nest of *Apis mellifera*. A model that uses infrared radiation for host location by the ectoparasite *Varroa* is proposed by Bruce (1997). The mite has appropriate sensory structures or setae located on the first pair of legs.

HEARING, VIBRATION AND AIR MOVEMENT PERCEPTION: In mites no sensory structures for detecting acoustic stimuli are known. If *Varroa* can detect sound with high acoustic amplitudes, then it does it with the help of mechanoreceptors sensitive to surface vibrations. The sensitivity to vibrations in *Varroa destructor* was proven to be comparable to the sensitivity of its host, the honey bee (Kirchner, 1993). If *Varroa* uses the substrate vibrations on the comb for its orientation is still unknown. The comb surface is especially suited as medium for the propagation of the honey bee dance language information (Tautz, 2002; von Frisch, 1965) in the form of substrate vibrations. Due to the mechanical dampening properties of wax, this information channel needs a very effective energy transfer as present in the bees' waggle dance (Tautz, 2002). *Varroa* is too small for an active use of this information channel. The *Varroa*

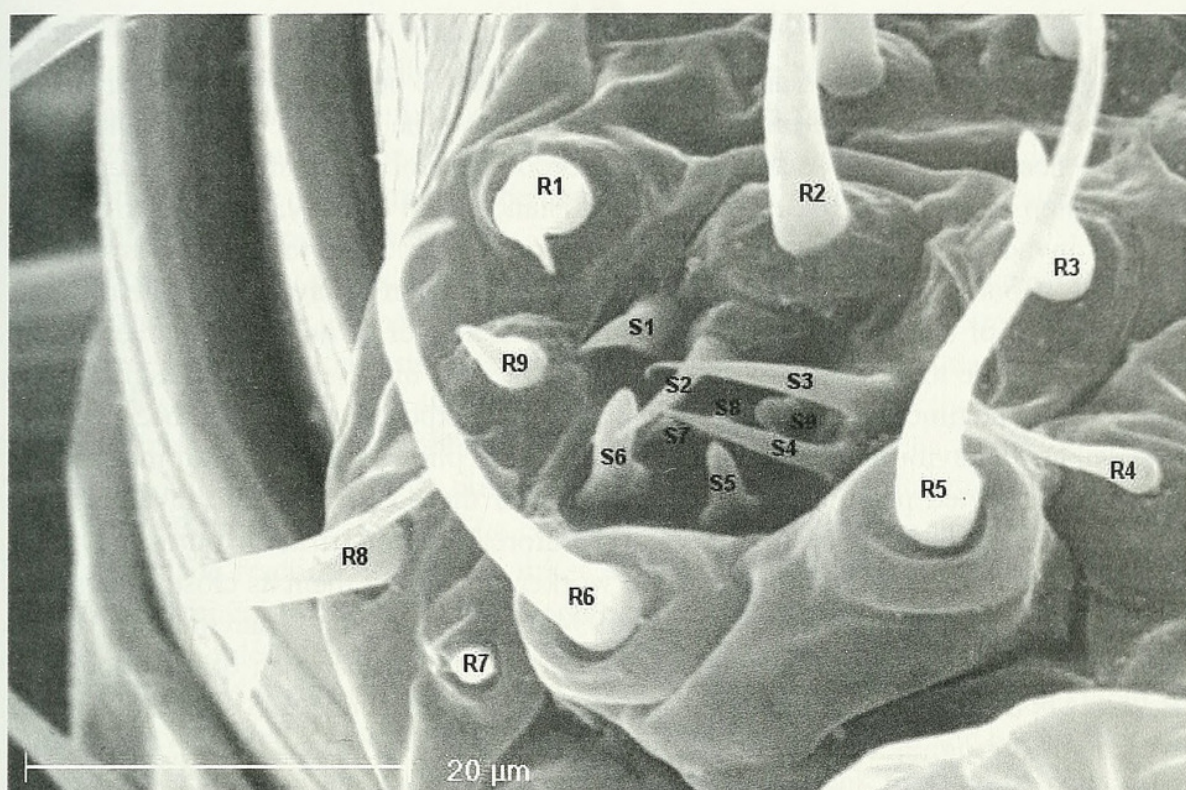


FIG. 2

Scanning 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 (SEM by M. Vlimant, University of Neuchâtel).

mites are very sensitive to movement of the air (Dillier *et al.*, 2002a; Kuenen & Calderone, 1998). The legs and also other body parts of *Varroa* are covered with mechanosensitive hair sensilla; possible receptors for the sense of touch as well as air movement (Fig. 2). The mite stops its translational movements and displays its frontal legs in the air in a typical manner for reorientation (Dillier *et al.*, 2002a; Rickli *et al.*, 1992; Rickli, 1994) in reaction to subtle air puffs. It has been shown by Kuenen and Calderone (1998) that *Varroa* can use single clean-air puffs in a wind tunnel as directional cues. *Varroa* turned nearly straight upwind in response to single 0.1-s puffs of clean air directed at 90 degrees to their anterior-posterior axis. This behaviour is probably part of the olfactory orientation system in *Varroa* and helps to add directionality to the volatile chemicals in an odour plume.

VISION: *Varroa destructor* has no eyes, but nevertheless behavioural responses to optical stimuli have been observed by Kirchner (1993). Part of the central nervous system, probably a small group of histaminergic cells, is light sensitive. Because there is no photoreceptor-like structure, the discrimination between different light source directions or shapes of objects is not possible. Low light sensitivity is hardly of much help in a specific orientation task outside a simple phototactic reaction.

HOST FINDING BEHAVIOUR IN VARROA

CELL INVASION: There are two crucial decisions to be made during cell invasion behaviour of female *Varroa* mites: first, when to leave the phoretic stage on the nurse bee and then, how to choose an appropriate brood cell for successful reproduction. In the first step the mite has to decide at some distance from the larva whether to stay on a bee or invade a cell and then in the second step in contact with the cell if it is suitable to invade (Boot, 1994). Direct observation of this invasion behaviour is quite difficult because most of the action takes place underneath a layer of nurse bees covering the brood nest in the hive. It is also suspected that there is high selective pressure on the mite to keep the time span for freely walking around exposed to the nurse bees as short as possible. Behavioural observations show that the phoretic mites spend most of the time in a protected position under the sternites of the host bee before invading host cells where they hide behind the larvae. Therefore *Varroa destructor* mites may only invade worker or drone brood cells when worker bees bring them as close as possible to a suitable brood cells (Boot *et al.*, 1994a). The invasion behaviour of *Varroa destructor* into brood cells of *Apis mellifera* was studied using an observation hive (Beetsma *et al.*, 1999; Boot *et al.*, 1990, 1992a, b, 1994a; Boot, 1995) and videotaping half combs through a transparent back wall (Beetsma *et al.*, 1993, 1999). It has been observed on video recordings that the mites moved from the bees to the rim of the cell, walked quickly inside, crawled between the larva and the cell wall, and then moved on to the bottom of the cell, hiding in the food juice (Boot *et al.*, 1992a; de Jong *et al.*, 1982; Donzé & Guerin, 1997; Ifantidis, 1988; Rath, 1991). According to Beetsma and collaborators (1999), *Varroa destructor* mites were never seen walking across the comb and entering and leaving brood cells as has been described for *Tropilaelaps clareae* (Delfinado & Baker) (Boot *et al.*, 1994a). Mites stay longer on bees if there are few brood cells available in the colony. Boot (1995) interprets this behaviour as minimisations of the period spend searching for a cell. But the few observations of walking mites could also result from the special lateral observation technique used by Beetsma and collaborators (1993) and the rareness of direct observations of the actual invasion. During many hours of observation of brood frames to collect infested *Varroa*, we repeatedly observed mites leaving nurse bees and running several centimetres over the comb surface before disappearing. Kuenen & Calderone (2000) also assumed that the mites leave their host and walk along the comb surface until they locate an appropriate larva or reacquire a nurse bee. They regularly saw *Varroa* mites walking on undisturbed comb surfaces in test colonies and on video tapes of observation colonies, walking along open cell rims often dipping into the lumen of a cell and then alternate to the other side to dip into the lumen of the next cell and so on.

There is also indirect evidence for an active search for appropriate brood cells by *Varroa* females from laboratory behavioural bioassay, developed to measure transfer of *Varroa* mites from nurse bees to the honey bee brood cells for reproduction (Dillier *et al.*, 2002a, b). After the test more mites were found on the comb side of the test box, in test series where the mites were prevented from returning to a nurse bee by a mechanical trap or an insect glue trap, than in control series (Dillier *et al.*, 2002b). This can be interpreted as a hint that a certain number of female mites always leave the

nurse bee to search actively for appropriate 5th instar larva cells. The situation in the hive remains unclear. A verification with a similar trap bioassay in test hives with a simultaneous choice between traps and unmanipulated brood was not possible (Dillier *et al.*, 2003). Because of the limited data available, it is not clear if the under representation of freely walking mites in behavioural records is a methodological artefact or the result of an ecological pressure.

For successful reproduction, the timing of the cell invasion is crucial. The mite invades the brood cell just before operculation. Only 5th instar larvae are attractive for the mites. This attraction lasts only for a short period prior to cell capping of 15-20 hours for worker brood and 40-50 hours for drone brood, respectively (Berg *et al.*, 1999; Blumer-Meyre *et al.*, 1998; Boot *et al.*, 1990, 1992a; Fuchs & Müller, 1988). The invasion of the mite into a brood cell is not related to the duration of its stay on adult bees (Boot *et al.*, 1995a). The condition of the hive has some influence on the infestation. The fraction of phoretic mites available in the hive which invades brood cells is determined by the ratio of the number of suitable brood cells and the number of bees in the colony (Boot *et al.*, 1994b, 1995c; Calis *et al.*, 1991). This means that the infestation of the brood cells does not only depend on the invasion behaviour of *Varroa*, but also on the amount and quality of the bee colony brood and on the strength of the worker force of the nurse bees. There is also a feedback from the *Varroa* infestation to the behaviour of the bees. A semiochemical from brood cells infested by *Varroa destructor* triggers hygienic behaviour in *Apis mellifera* (Nazzi *et al.*, 2004a).

PREFERENCES FOR AGE AND SEX OF THE HOST LARVAE: In experiments only the 5th instar larvae were infested by the mites. Several authors have reported preferences for drone cells over worker cells in the beehive (Boot *et al.*, 1992a, 1994b, 1995c; Calderone & Kuenen, 2001; Fuchs, 1990; Fuchs & Langenbach, 1989; Grobov, 1977; Ifantidis, 1984; Issa *et al.*, 1984; Le Conte *et al.*, 1989; Otten & Fuchs, 1988; Schulz, 1984a, b; Sulimanovic *et al.*, 1982; Vandame, 1996) or in laboratory bio-tests (Dillier *et al.*, 2002a; Rosenkranz *et al.*, 1984; Tewarson, 1983; Zaitoun, 1993).

The attractive period of drone brood cells is two to three times longer than that of worker brood cells (Boot *et al.*, 1992a; Calis, 2001). The distribution of mites over worker- and drone-brood in a colony is determined by the brood type specific rates of invasion and the numbers of both brood cell types. Drone brood cells were invaded 11.6 times more frequently than worker brood cells (Boot *et al.*, 1995c). Drone larvae produce greater quantities of attractive compounds (esters attractive to *Varroa*) and they produce them over a longer period of time (Calderone & Lin, 2001; Le Conte *et al.*, 1989). This preference could be partially due to the 1.7 fold larger cell surface of a drone cell (Boot *et al.*, 1995b), the 2-3 times longer attractive period of drone larvae (Boot *et al.*, 1991, 1992a, 1995b; Fuchs & Müller, 1988), and the larger size of drone larvae compared to worker larvae (346 mg and 147 mg respectively) (Beetsma *et al.*, 1999; Martin, 1998). But these effects are interdependent and not additive because in drone larvae cells as well as the larvae are already bigger in a younger developmental stage. There seems to be a still higher attractiveness of the drone larvae itself, stemming from differences in chemical recognition factors (Le Conte *et al.*, 1989, 1991, 1994; Trouiller *et al.*, 1991, 1992).

In behavioural bioassay experiments with two compartment polystyrene test boxes (Dillier *et al.*, 2002a), where either 5th instar worker or 5th instar drone larvae were presented in parallel tests, *Varroa* moved to the brood more often when drone instead of worker larvae were available on an standardized comb surface. This implies that there must be a higher motivation to leave bees in the proximity of drone larvae than in the proximity of worker larvae, or to stay longer on the comb side after having left the bees in the test situation.

Nurse bees also visit drone larvae more frequently, because they receive more food than worker bees Fuchs (1990).

Mite prevalence values in queen cells were 15 times smaller than in worker cells (Calderone *et al.*, 2002; Harizanis, 1991), where the development time of only 8.0 to 8.5 days in the capped cell stage is too short (*A lecture given to the Central Association of Bee-Keepers*) for successful *Varroa* reproduction (Rehm & Ritter, 1989; Romaniuk *et al.*, 1988). This could be caused by the fact that royal jelly contains a repellent to *Varroa* (Le Conte, 1990). These results raise the question of the origin of the cues responsible for age and sex specific differences in the brood infestation.

CHEMICAL ORIENTATION CUES: Behavioural bioassays in the laboratory of Rosenkranz (1990) do not confirm the presence of a stage- or sex-specific kairomon in bee larvae. A characterisation of the cuticular hydrocarbons of *Varroa* mites and their honey bee hosts by gas chromatography (GC) and coupled GC-mass spectroscopy (MS) (Nation *et al.*, 1992) found no qualitative differences in the chromatographic spike pattern between the mites and the host bee larvae. More likely are quantitative variations in the composition of some substances in the cuticle. The sex of the host was not an important factor affecting the behavioural responses of the mites in any assay (Calderone & Lin, 2001). They concluded that host kairomones play a role in the host acquisition process, but found no evidence to support the hypothesis that mites use such substances to differentiate between worker and drone brood.

More detailed analyses in different laboratories revealed the existence of sex- and age-specific differences, mainly in the quantitative composition of complex mixtures of compounds, even within the bee larvae. Living drone and worker larvae differ in their profile of volatiles analysed by means of Purge and Trap/Gas chromatography (Purge and Trap system LSC3100, Tekmar Cincinnati USA) (Dillier *et al.*, 2002a) and in cuticular volatile hydrocarbon profiles from bee larval extracts. Aumeier (Aumeier, 2001; Aumeier *et al.*, 2002; Chiroudi *et al.*, 1997) detected age- and caste-specific differences and Troullier and collaborators (1994) found characteristic quantitative differences especially in the esters present on worker, drone and queen brood.

It has been shown that *Varroa* can use bee-odour plumes in a wind tunnel as directional cues (Kuenen & Calderone, 1998). When released in odour plumes and control plumes mid-way between the plume's origin and the downwind end of the tunnel, mites responded to bee-odour by walking upwind in a zigzag fashion, or by walking along the edge of the odour plume. In the wind tunnel (Calderone & Lin, 2001) mites were attracted to odours from living 5th instar worker and drone larvae, but not to volatiles from cocoons, brood food or a blend of fatty acid esters found to be attractive in other bioassays (see below). Using a four-arm airflow olfactometer, also

le Conte and collaborators (Le Conte *et al.*, 1989, 1991; Le Conte, 1990; Trouiller *et al.*, 1992, 1993, 1994) found that *Varroa* is attracted by odour to its preferred host, "drone larvae". The airflow containing the odour of either drone larvae or a total n-hexane extract of drone larvae was effective. The identified fractions and pure compounds were tested again with the same olfactometer set-up. Three methyl and ethyl esters of straight-chain fatty acids induced attraction (Le Conte *et al.*, 1989), in particular methyl palmitate but also ethyl palmitate and methyl linolenate.

In a bioassay the movement of individual mites on the surface of a servosphere was recorded while different odours were released at the top of the sphere (Rickli *et al.*, 1992; Rickli, 1994). The most attractive odour was that of living larvae, closely followed by larval headspace extract. The odour of live bees was also attractive, but to a lesser degree. Methyl palmitate, which has previously been reported to be attractive to mites (Le Conte *et al.*, 1989), was moderately attractive on the servosphere. However, palmitic acid, the precursor of this ester, was as attractive as were living larvae.

Wind tunnels and olfactometer tests as well as the servosphere are rather unnatural environments for *Varroa destructor*. The mites are not adapted to run over long distances. They tend to stop and try to hide themselves under a cover or in a depression of the surface or in a crack in reaction to any kind of disturbance. If hiding is not possible, the mite tries to jump away. Therefore tests for orientation cues in a situation more similar to the hive conditions are needed to confirm these results.

Zetlmeisl & Rosenkranz (1994) found in laboratory bioassays that dummies treated with methyl palmitate, ethyl palmitate and methyl linolenate had no effect on mites, but when put in hives, capping behaviour by workers was observed. This confirms that these esters have a pheromone effect on bees but the kairomone function on mites remains unclear.

Cuticle extracts of 8-day-old worker honey bee larvae occupying brood cells have an arrestment effect on the mite (Rickli *et al.*, 1994). Extracts induce a strong arrestment response in the mite, as indicated by prolonged periods of walking on the extracts applied to a semi permeable membrane stretched over a water bath to provide high relative humidity. Mites increased walking speed and path straightness in response to increasing doses of a nonpolar fraction of the cuticle extract. Saturated straight-chain odd-numbered C-19-C-29 hydrocarbons were identified by thin-layer argentation chromatography and gas chromatography-mass spectrometry as the most active constituents, with branched alkanes also contributing to the arrestment effect of this active fraction.

Aliphatic alcohols and aldehydes of the honey bee cocoon also induced arrestment behaviour in *Varroa* (Donzé *et al.*, 1998) in this test. Two thin-layer chromatography fractions of the cocoon extract eliciting arrestment were found to contain saturated C-17 to C-22 primary aliphatic alcohols and C-19 to C-22 aldehydes. Both aldehydes and alcohols were more abundant in the cocoon than in the cuticle of adult or developing bees. Analysis of the behavioural responses to synthetic n-alkanes indicates that the response is probably based on a synergism between the different alkane components of the fraction rather than on an individual compound. When these oxygenated chemo stimuli were mixed with C-19 to C-25 alkanes at the proportions found in the cocoon extract, a significantly lower threshold for the chemo stimulant mixture was observed.

In a contact bioassay on glass Calderone and collaborators (Calderone *et al.*, 2002; Calderone & Lin, 2000; 2001) found arrestment responses of mites to solvent extracts of the cuticula of worker and drone bee larvae obtained 0.24 and 48 hours prior to cell capping, to brood food and cocoons, and to a blend of synthetic fatty acids that were found to be attractive (Le Conte *et al.*, 1989; Le Conte, 1990; 1991; Trouiller *et al.*, 1992; 1993; 1994). There was no difference in the arrestment behaviour between extracts of worker and drone larvae but less arrestment with extracts of queen larvae (Calderone *et al.*, 2002; Calderone & Lin, 2001). Calderone and collaborators detected a repellent effect of royal jelly on *Varroa* but no arrestment or repellent effect of worker jelly or drone jelly. Findings of these authors (Calderone *et al.*, 2002; Calderone & Lin, 2001) suggest that the low incidence of mites in queen brood is in part due to the repellent activity of royal jelly, and possibly to intrinsic differences between larval chemistries.

Zaitoun (1993) found that nurse bees in contact with open brood of any age attract *Varroa*. Adult bees are at least as attractive as larvae (Kraus *et al.*, 1986; Kraus, 1993). Mites could distinguish between nurse bees, pollen foragers and larvae in simultaneous choice tests in Petri dishes, but tests in a Y-shaped olfactometer showed no clear difference in preferences for bees of different ages (Kraus, 1993, 1994). This contrast could stem from the different set-up of experiments. It is an indication that in choice tests not only volatiles but also less volatile cues or contact stimuli could play a role. The preference for nurse bees or for larvae and forager bees was dependent of the source of the mites, if they were kept before on nurse bees, larvae or forager bees. Phoretic mites from bees prefer again bees to larvae. The host preference is therefore not only influenced by factors stemming from the future host but also by past experience or intrinsic factors that depend on the female mite's reproductive stage.

Rosenkranz (1993) developed a laboratory choice test for preferences of *Varroa* females for different types of bee larvae, adult bees, or cuticular extracts on dummies. This test, in a closed arena with short distances of less than 2 cm ("Rosenkranz arena"), better corresponds to the natural situation in the beehive. When offered a choice between 5th instar larvae and adult workers phoretic *Varroa* again showed a preference for adult workers (Zetlmeisl & Rosenkranz, 1994). This bioassay confirmed the preference of *Varroa* females for certain developmental host stages and their corresponding extracts (Aumeier *et al.*, 2002). Although it was found in the laboratory bioassay that individual *Apis mellifera carnica* larvae were less attractive than Africanized larvae (Aumeier, 2001; Aumeier *et al.*, 2002; Aumeier & Rosenkranz, 1997), the infestation rate of *Apis mellifera carnica* in hive experiments was 2 to 6 times higher. The authors suggested that the quantity and composition of certain cuticular compounds are responsible only for the recognition of a suitable host stage by *Varroa* females (Aumeier, 2001; Aumeier *et al.*, 2002). There must be other factors that are also important for cell invasion behaviour, especially in the second step of the Boot-model (1994), to decide whether a larva cell is suitable for invasion.

In a similar bioassay larval food collected from drone cells before capping elicited a strong response from *Varroa* (Nazzi *et al.*, 2001). Both ether and acetone extracts of larval food induced the same response as larval food itself, thus suggesting the existence of semiochemicals attracting or arresting the mite. Linear, branched and

aromatic carboxy-acids, as well as hydroxy-acids, were identified by GC-MS and SPME-GC-MS analysis in organic larval food extracts. Loss of activity after neutralization and air entrainment experiments suggested that the active substances were acidic and volatile. The carboxy-acids of low molecular weight identified were tested in a laboratory assay using a "Rosenkranz-arena". All the acids tested were inactive, with the exception of 2-hydroxyhexanoic acid. One hundred nanograms of 2-hydroxyhexanoic acid applied to worker brood cells before capping increased the number of mites per cell in the treated brood by 36% in comparison to the control cells (Nazzi *et al.*, 2004b).

There is an enhanced probability that *Varroa* is found on the comb side of test boxes of brood cells with living larvae or food jelly, contrary to clean empty cells (Dillier *et al.*, 2002a, 2002b). This demonstrates that cues associated with brood are involved in the decision by female mites to either leave host bees or to stay on the comb side. In tests where either 5th instar worker or 5th instar drone larvae were presented in parallel tests, *Varroa* decided to move to the brood more often than when drone larvae were available (Dillier *et al.*, 2002a, b). This implies that there must be a higher motivation to leave bees in the proximity of drone larvae than of worker larvae or to stay longer on the comb side after having left the bees.

In her experiments on brood attractivity in small test hives, Zaitoun (1993) found that a separation of the two compartments by more than 2 cm was enough to stop the transfer of mites between the compartments. The author concluded that the mite has to come in close contact with appropriate brood or cues transmitted by the nurse bees from the larvae to the mites. The aliphatic esters and their mixtures found attractive in the olfactometric tests (Le Conte *et al.*, 1989, 1991; Le Conte, 1990; Trouiller *et al.*, 1992, 1993, 1994) showed no effect in this set-up. The four fatty acid methyl esters (methyl palmitate, methyl oleate, methyl linoleate and methyl linolenate), secretions which are present on the surface of worker and drone larvae only a few hours before the cell is closed, also trigger the capping of brood cells by worker bees (Le Conte *et al.*, 1990b; Trouiller *et al.*, 1991, 1992).

Some of these observations from different laboratories suggest the possibility of an indirect transfer of information about the status of the brood, where the nurse bees act as intermediary. If *Varroa* uses the fatty acid methyl ester secretions on the cuticle of bee larvae as a cue for the timing of its cell invasion behaviour, then it is somehow "hitchhiking" on the information network of the honeybees.

INFLUENCE OF PHYSICAL PARAMETERS OF THE COMB ON *VARROA* INFESTATION

Under experimental fostering conditions in the broodless hive of a drone eggs laying Africanised honey bee queen (or of drone eggs laying workers) drone larvae in drone sized cells are significantly more heavily infested by *Varroa* mites (0.38 mites/larva) than in worker sized cells (0.188 mites/larva) (Cavicho Issa *et al.*, 1993). But the effect of cell size was outdone by the effect of the type of larvae in the cross fostering experiment of drone larvae in worker sized cells (1.86 mites/larva) and worker larvae in drone sized cells (0.379 mites/larva). Drone larvae are more attractive

than worker larvae, even in combs with the inappropriate cell size. A similar pattern was found by Calderone & Kuenen (2001) in a cross fostering experiment with non-Africanised honey bees: the highest mite infestation levels were found in drone larvae reared in drone cells, followed by drone larvae reared in worker cells, worker larvae reared in worker cells and worker larvae reared in drone cells. This could not be interpreted as an indication of a pure size effect, but an interaction between larval sex and cell type. To complicate the issue even further, there is an additional strong colony effect on the level of this interaction, and small cells have a significant negative effect on the weight of 5th-instar drones, but large cells have no positive effect on worker larvae.

In empty cell frame transfer experiments with Africanised bee hives a 2.6 times higher infestation rate by *Varroa* of the Africanised bee brood was observed in the larger cells built by European bees, *Apis mellifera ligustica*, compared with smaller cells built by bees of their own race (11.9% versus 4.7% of the cells with female deutonymphs infested with *Varroa* respectively). This is an indication for an influence of the cell size (Message & Goncalves, 1995).

In manipulated (shortened) cells also larvae normally not attractive do attract mites (Boot *et al.*, 1995b). Cells with artificially raised, wax filled cell bottoms (de Ruijter & Calis, 1988) attract more mites. Cells shortened by placing a grid in front of the comb and restricting the builder bees to finish the cell construction (Goetz & Koeniger, 1993) are already attractive earlier, even though they contain larvae of a smaller size than normal ones. However, this effect is counterbalanced by the fact that the shorter distance to the cell rim also triggers the bees to begin cell-capping behaviour earlier. If the distance from the cell rim to the larvae was larger than in the control cells with larvae of the same age, the attractive period was shorter and vice versa (Beetsma *et al.*, 1999; Boot *et al.*, 1995b, c). There seems to be a critical larva-to-rim distance under which brood cells are attractive to mites. The maximal distance is 6.9 to 7.9 mm for natural cells, but longer (8.2 to 9.0 mm) for elongated worker cells or drone cells containing older worker larvae (Beetsma *et al.*, 1999). The number of *Varroa* mites found in the capped brood cells seems to be determined by the duration of the attractive period. The length of this period results from an interaction of the signal that triggers capping behaviour of the bees and the cues responsible for the attraction of the *Varroa* to the larvae.

The duration of the post capping stage, in which the bee larvae develop, and the proportion of the infertile mites in infested cells are not influenced by cell size (Fries, 1994). According to different observations (Boot *et al.*, 1995b; Calis *et al.*, 1993; Ramon *et al.*, 1993), small cells are much longer attractive to the mites and therefore accelerate the population dynamics of the *Varroa* infestation.

Natural cell size is a reliable race specific trait (Spivak & Erickson, 1992) not much influenced by the size of the commercial cell frame foundation on which the colonies were reared before natural comb building. This was confirmed by Piccirillo & de Jong (2003), who showed the brood cell infestation rates were significantly higher in the Carniolan-sized (5.27 mm, built naturally by Carniolan bees) comb cells than in the Italian (5.16 mm, that the bees made from Italian-sized commercial foundation) and Africanized cells (4.84 mm). European-sized worker brood cells were always more

infested than the Africanized worker brood cells in the same colony (19.3% Carniolan cells, 13.9% Italian and 10.3% Africanized, respectively).

Because *Varroa* preferentially invades larger cells under experimental conditions with Africanized bees, it was expected that brood in old combs with reduced-size cells would be less infested than the brood in new comb cells (Piccirillo & de Jong, 2004). However in European beehives small cells are usually longer attractive to *Varroa* (Boot *et al.*, 1995b; Calis *et al.*, 1993). Indeed the percentage of brood cells infested in Africanized honey bee colonies was significantly higher in the old combs (22.6%) than in the new combs (9.75%), even though the inside width of the cells was significantly smaller in the old (4.58 mm) than in the new combs (4.85 mm) (Piccirillo & de Jong, 2004). But also some factor other than cell size, perhaps olfactory or gustatory cues from the cocoons (Calderone & Lin, 2001) or cell walls, can make old brood comb cells much more attractive to *Varroa destructor* than newly constructed brood combs. The study of Taylor (2002) on European bee nucleus colonies shows no significant difference in the infestation rates on frames with a mosaic of five different cell sizes (4.7, 4.8, 5.0, 5.1 and 5.4 mm cell diameter). The highest infestation level (46.6%) was not found in the biggest but in the rather small (4.8 mm) cells.

The inconsistent results from different studies indicate that cell size is not the decisive key factor in the invasion behaviour. The question remains whether slightly smaller cell diameters of naturally built combs, as compared to cells that bees made from commercial foundations provided by the beekeepers, can reduce the parasite load in hive conditions. It is also unclear if this effect can be observed only in Africanised honeybee colonies and/or if this phenomenon is coupled with other behavioural or physiological characteristics of these hybrid bee strains.

There is also evidence that the raised cells are more infested by *Varroa* (de Ruijter & Calis, 1988) and the raised borders of drone cell patches contribute to the clumped distribution of infested brood cells on mixed worker drone brood combs (de Jong & Morse, 1988; Kuenen & Calderone, 2000). It is still unclear if raised borders influence the behaviour of the nurse bees or if this is an effect of catching *Varroa* mites searching on the comb surface for suitable cells at the borders of raised cell patches. There is a 6 fold higher infestation of the edge cells of the raised patch, but not of the interior cells of the patch.

CONCLUSIONS

The orientation of *Varroa* and its cell invasion behaviour is certainly a key factor for the infestation of honeybee colonies. *Varroa* is equipped for orientation with a variety of senses, ranging from olfactory detection of volatiles over a certain distance to the contact senses of touch and taste. Research on *Varroa* orientation has mainly focused on the chemical senses olfaction and gustation. Perhaps this has led to an underestimation of the role of other sensory modalities like temperature, vibration and unspecific orientation cues like humidity or CO₂ concentration.

Calderone & Lin (2001) discussed four hypotheses on how female *Varroa* mites are guided to the appropriate brood cells for reproduction: chemical cues, physical parameters, interaction of chemical- and physical cues and passive transport by the nurse bees.

NURSE BEE BEHAVIOUR AND MOBILITY OF PHORETIC *VARROA*: Beetsma and collaborators (1999) postulated a passive transport of the female *Varroa* mites close to the appropriate brood cells by nurse bees, where the active cell invasion of the mite has only to cover a very short distance (of some Millimetres). In laboratory test situations, however a rather high mobility of *Varroa* females was observed. In box tests with glue and Fluon traps (Dillier *et al.*, 2002b) up to one third of the phoretic *Varroa* left the bees within 24 h. It is not clear yet if part of this unexpected active role of *Varroa* in the searching behaviour (Kuenen & Calderone, 2000) is due to the specific test situation or if it belongs to the normal behaviour of the species under natural conditions.

CHEMICAL HYPOTHESIS: A variety of extracts and compounds have been tested for their behavioural activity. A compilation of the assays and the tested substances is presented by Meier (1998) and Aumeier and collaborators (2002). Not single compounds but rather mixtures of them seem to provide the more active cues. Results on synergistic effects in arrestment behaviour caused by mixtures of rare saturated C-17 to C-22 primary aliphatic alcohols and C-19 to C-22 aldehydes with higher quantities of C-19 to C-25 alkanes indicate how *Varroa* may use mixtures of rarer products to differentiate between substrates and host stages during its developmental cycle within honey bee brood cells (Donzé *et al.*, 1998). Most results indicate that *Varroa* uses quantitative instead of qualitative differences in chemical cues presented by the bee larvae to determine their sex and age.

INTERACTION WITH PHYSICAL PARAMETERS: A variety of parameters of the comb, such as cell size, raised cell rims, shortened or elongated cells, have some influence on the infestation rate of the brood cells. Preference for cracks and narrow spaces, thigmotaxis, as expressed by the mites behaviour to hide between the folds of the abdominal segments of host bees (Ifantidis, 1988), could help the mites to find cells with large enough larvae to hide behind them. However the relative importance of these cues and their role in the orientation process remain unclear. Calderone & Kuenen (2001) stated that there is an interaction between physical and chemical effects, such as honey bee colony, cell type and larval sex, influencing the cell invasion behaviour of *Varroa destructor*. All together this can lead to the 5.5 to 12 times higher infestation rate of drone brood cells compared to worker brood cells in *Apis mellifera* (Martin, 1998) and to the precise timing of invading brood cells in the beehive.

The cell invasion behaviour in *Varroa destructor* seems to be influenced by a mixture of multiple factors and cues, which are detected through different sensory pathways. Test results produced under laboratory conditions need confirmation in the more complex hive situation before we can understand their relevance in the host-parasite interaction of *Varroa destructor* and *Apis mellifera*. Most likely the chemical contact sense (taste) plays an important role in the decision whether to stay in the chosen brood cell or to return to a bee, but the whole set of available sensory modes may be involved in the form of a "Gestalt".

Any crude intervention in the host-parasite interactions risks to disturb the fine tuned information network of the beehive too much. In consideration of the present state of knowledge, there is no practicable biological *Varroa* treatment regime in sight for beekeepers based on *Varroa* orientation. For a deeper insight in the host-parasite interaction a multidisciplinary approach is needed.

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