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Methyl palmitate does not elicit invasion of honeybee brood cells by *Varroa* mites.

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ABSTRACT

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Key words: Methyl palmitate, *Varroa jacobsoni*, invasion behaviour.

Since *Varroa* mites decide at some distance from the larva whether to stay on a bee or invade a cell, they may well use a volatile chemical to select a brood cell. A few aliphatic esters, predominantly methyl palmitate, have been claimed to be this volatile signal for the mites for two reasons. The mites respond to the esters in an olfactometer (Le Conte *et al.*, 1989), and the levels of the esters in worker and drone larvae may explain that drone cells are attractive during a longer period and are invaded more frequently than worker cells (Trouiller *et al.*, 1992). However, invasion itself appeared to be unaffected by application of methyl palmitate to brood cells. In addition, analysis of the volatiles emanating from attractive brood cells showed hundreds of components in the volatile blend, but in only 2 of 17 analyses a trace of methyl palmitate was found. Hence, there is no reason to believe that methyl palmitate is used as a signal for invasion by the mites.

INTRODUCTION

Varroa jacobsoni Oudemans, a major acarine pest of the honey-bee, *Apis mellifera* L., can only reproduce in capped brood cells (Ifantidis and Rosenkranz, 1988). Outside the brood cells, *Varroa* mites stay exclusively on adult bees. In undisturbed colonies, the mites do not walk over the comb, nor do they leave a cell once they have entered it. They are carried close to a suitable cell by a bee, and move directly from the bee into the cell (Boot *et al.*, 1994).

Varroa mites invade worker and drone cells during a period of 15–20 h and 40–50 h preceding cell capping respectively (Ifantidis, 1988; Boot *et al.*, 1992). They may obtain information about the content of the cell to determine whether to stay on the bee or to invade a brood cell, *e.g.* by using volatile chemicals. Recently, it has been found that the response of the mites is correlated with the distance from larva to cell rim (Boot *et al.*, in press). For example, cells that had been shortened were

invaded earlier than control cells containing larvae of the same age. The results suggest that the distance from larva to cell rim affects the strength of a signal reaching the mites on bees, and that invasion occurs when the strength of this signal passes a threshold.

Mites invade drone cells with a 12 times higher rate than they invade worker cells (number of mites per cell and per day; Boot *et al.*, unpublished). This higher invasion rate partly results from the 2–3 times longer attractive period of drone cells. Additionally, a 1.7 times higher invasion rate is expected if the invasion rate is proportional to the area on the comb's surface of a brood cell. Taken together, this would result in a 3.4–5.1 times higher invasion rate, much less than the rate actually found (Boot *et al.*, unpublished), which is 12 times higher. Thus, the higher invasion rate into drone cells may be mainly attributed to quantitative or qualitative differences in the signal to invade between worker and drone cells.

Le Conte *et al.* (1989) claimed that the signal for invasion of both worker and drone cells consists of the odour of a few aliphatic esters, especially methyl palmitate. They showed that each of these esters, which had been extracted from the larval cuticle, attracted mites in an olfactometer. A maximum of 17 ng and 320 ng of methyl palmitate can be extracted from the cuticle of a worker or drone larva respectively (Trouiller *et al.*, 1991). Since drone larvae secrete much more of the esters, and these are secreted during a longer period preceding cell capping (Trouiller *et al.*, 1992), secretion of the esters correlates with the differential invasion of worker and drone cells by the mites. Thus, methyl palmitate may well be a signal used by *Varroa* mites for invasion.

In the study of Le Conte *et al.* (1989), the response of mites to methyl palmitate was determined in an olfactometer in which the mites walked on a flat surface of 56×56 mm. Such a bio-assay only demonstrates that mites can respond to this ester, but not whether they use the ester to decide to enter a cell or stay on a bee. In the present study, methyl palmitate was applied to worker cells to determine whether it actually affects invasion by *Varroa* mites. Both the number of mites found per treated cell, and the length of the attractive period of treated cells were compared with that of control cells. If methyl palmitate is a primary signal to invade, it is expected that application will increase the number of mites per cell and that cells will attract mites over a longer period.

MATERIALS AND METHODS

Experiments were carried out in 10-frame hives with honey bees commonly used in the Netherlands. Special 'half-combs', from which the bottoms of the cells had been replaced by a transparent sheet, were used (Beetsma *et al.*, 1993). Two 'half-combs' were clamped together and placed in the middle of a bee colony heavily infested with *Varroa* mites (Boot *et al.*, in press). One of the 'half-combs' contained a patch of open worker brood with larvae varying up to one day in

age (Boot and Calis, 1991). No other combs with open brood were present in the colony.

To determine the attractive period of treated and control worker cells, the test comb was removed from the colony, the adhering bees were brushed gently into a bucket, the two 'half-combs' were separated, and the patch of brood was held against the light. Most mites could easily be seen through the transparent cell bottoms (*ca* 10% is usually overlooked in this kind of experiment [Boot *et al.*, in press]). This inspection was repeated at two-hour intervals. For each cell, records were made of when a mite had appeared and when the cell had been capped. These data were later used to calculate when each mite had invaded in terms of the number of hours preceding cell capping. After inspection, the 'half-combs' were clamped together, returned to the colony, and the bees collected in the bucket were replaced by shaking them on top of the test comb.

Treatment of cells commenced after one or a few cells of the patch of brood had been invaded. Into each of a group of about 100 cells a droplet consisting of 2 μ L of 10, 1 or 0.1% methyl palmitate dissolved in acetone (v/v) was applied (Wirtz, 1973). This corresponds to an additional 172, 17.2 or 1.72 ng methyl palmitate in the treated worker cell respectively.

The number of mites invaded per cell were also compared between treated and control cells. Since the number of mites that reside on adult bees may vary strongly over time in a bee colony, the number of mites per cell should be compared between cells that attracted mites during the same period. Therefore, the cells were grouped in intervals of 4 hours according to their capping time, and each interval was treated as a pseudo-replicate. Only those 4-hour intervals during which 10 or more mites had invaded were used to compare the number of mites per cell. This resulted in 4 intervals in all trials. For each interval the ratio of the number of mites per treated cell to the number per control cell was calculated. The average ratio was calculated from the ratios in the separate intervals, weighted to the number of mites that had invaded.

RESULTS

Since methyl palmitate was dissolved in acetone, tests were first carried out to determine whether acetone itself affected invasion of the cells by the mites. A droplet of acetone (2 μ L) had no effect on either the attractive period of the cells (Table 1), or the number of mites per cell (Table 2). Application of methyl palmitate did not affect invasion of the cells either (Tables 1 and 2). The only difference between treated and control cells was found in one of the trials in which 0.1 % methyl palmitate was applied. In this trial, a larger proportion of mites invaded 0–6 h preceding cell capping in comparison with the control, whereas the length of the attractive period was similar.

Applying high doses of methyl palmitate resulted in mortality of the larvae. When cells were treated with 10% methyl palmitate all larvae in the treated cells

TABLE 1

Attractive period of worker cells treated with acetone or methyl palmitate dissolved in acetone and control worker brood cells. The attractive period was defined as the period preceding cell capping during which 90% of the mites invaded.

Treatment ¹	Number of mites that invaded	Attractive period (hours)
Acetone ^{NS}	46	10
Control	32	12
Acetone ^{NS}	21	14
Control	123	12
Me-palmitate 1% ^{NS}	25	14
Control	108	14
Me-palmitate 0.1% ^{NS}	64	12
Control	108	14
Me-palmitate 0.1%**	61	12
Control	40	12

¹Differences in the distribution of invasions over time before cell capping between treated and control cells were tested with the Kolmogorov-Smirnov Two Sample test; NS = not significant; ** = $P < 0.01$

TABLE 2

Ratio between the number of mites that invaded per treated cell and the number that invaded per control cell¹.

Treatment	Total number of treated cells capped	Total number of control cells capped	Total number of mites	Average ratio to control cells ¹
Acetone	77	120	70	0.93 ^{NS}
Acetone	31	142	152	0.80 ^{NS,2}
Methyl palmitate 1%	49	127	103	0.56 ^{NS}
Methyl palmitate 0.1%	90	171	165	1.10 ^{NS}
Methyl palmitate 0.1%	44	48	94	1.50 ^{NS,3}

¹The number of mites were compared in 4 intervals of 4 hours. The average ratio was calculated from the ratios in the separate intervals, weighted to the number of mites that had invaded. Differences in number of mites per cell between treated and control cells were tested with Chi²-test; NS = not significant.

²When the data from the two Acetone treatments are combined the average ratio is 0.82 and there is no significant difference in the number of mites per cell ($0.3 < P < 0.5$).

³When the data from the two 0.1% Methyl palmitate treatments are combined the average ratio is 1.27 and there is no significant difference in the number of mites per cell ($0.3 < P < 0.5$).

died, either directly because of the treatment or indirectly because the bees no longer nursed the brood. Treatment with 1% methyl palmitate also caused some mortality: 1/3 of the larvae died in one trial, and 73 out of 75 larvae died in the other. Treatment with 0.1% methyl palmitate or acetone alone did not cause mortality.

DISCUSSION

The results do not support the suggestion that *Varroa* mites use methyl palmitate as a signal to invade brood cells of the honey bee, because application of this substance to brood cells did not increase the number of mites per cell, nor did it prolong the attractive period of the cells. In theory, methyl palmitate could still be one of the components of a composite signal, whereas it has no effect when offered alone. Furthermore, it cannot be excluded that the bioassay used was unable to show an effect. However, there is other evidence that methyl palmitate does not play a role in brood cell invasion. Mites select brood cells while on adult bees (Boot *et al.*, 1994). Thus, a signal from the larva has to bridge at least the distance from larva to cell rim, which measures 5–8 mm in attractive brood cells (Goetz and Koeniger, 1993; Boot *et al.*, in press). Therefore, a chemical that is used by the mites as a signal is expected to be volatile. Analysis of the volatiles emanating from attractive brood cells (*c.f.* Dicke *et al.*, 1990 for methodology), showed hundreds of components in the volatile blend, but in only 2 out of 17 analyses was a trace of methyl palmitate found (M.A. Posthumus and W.J. Boot, unpublished). Thus, both the experiment described here and the analysis of volatiles suggest that methyl palmitate is of minor importance as a signal for invasion.

Lc Conte *et al.* (1990) showed that methyl palmitate is used by the bees as a pheromone evoking capping behaviour. When paraffin lures impregnated with methyl palmitate were placed in cells, these cells were capped by the bees. In the experiment described here there was no evidence to suggest that treated cells were capped earlier than control cells. It is possible, however, that the doses of methyl palmitate applied to the cells may have been too low.

The bio-assay described here may be used to test whether chemicals other than methyl palmitate do affect invasion of brood cells. Some components of the volatile blend coming from brood cells have been tested already (W.J. Boot, unpublished), but so far no attractant has been identified. However, application of 2 volatile components emanating from drone larvae, capro-lactone and valero-lactone, resulted in a greatly reduced number of mites per cell.

The bio-assay method described here has some advantages over an olfactometer. It tests actual invasion behaviour of the mites whereas the olfactometer merely shows whether the mites respond to a specific odour. In addition, looked at the view-point of a *Varroa* mite, there are two separate problems concerning invasion of a cell: (1) how to identify whether or not a cell contains a larva, and subsequently (2) how to determine whether a cell is suitable to invade. Different stimuli may be involved in solving these two problems, and lack of stimuli indicating that a larva is present may prevent a response to stimuli indicating that a cell is suitable. If so, stimuli indicating that a cell is suitable to invade can still be tested because substances are applied to brood cells and this implies the presence of stimuli identifying larvae.

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