Apidologie 32 (2001) 149–155

Original article

Semiochemicals from larval food affect the locomotory behaviour of *Varroa destructor*

Francesco NAZZI*, Norberto MILANI, Giorgio DELLA VEDOVA, Matteo NIMIS

Dipartimento di Biologia applicata alla Difesa delle Piante, Università di Udine, via delle Scienze 208, 33100 Udine, Italy

(Received 27 July 2000; revised 11 January 2001; accepted 12 January 2001)

Abstract – The stimuli inducing cell invasion by *Varroa destructor* were studied using a bioassay in which a mite was observed in a glass arena with four wells, each containing a live bee larva, treated or not with the stimulus to be tested. Larval food collected from drone cells before capping elicited a strong response from *V. destructor*. Both ether and acetone extracts of larval food induced the same response as larval food itself suggesting the existence of semiochemicals attracting or arresting the mite.

***Varroa destructor* / semiochemicals / larval food / brood cell invasion**

1. INTRODUCTION

To reproduce, the mite *Varroa destructor* Anderson and Trueman enters a brood cell containing a bee larva 15–20 or 40–50 hours (for worker and drone brood, respectively) preceding cell capping (Boot et al., 1992).

Several authors have investigated the stimuli that trigger cell invasion by the mite. Le Conte et al. (1989) showed that the mite is attracted by some methyl and ethyl esters of simple aliphatic fatty acids found in crude extracts of fifth instar larvae (*l5*); among them methyl palmitate which gave the strongest attractive response at the concentration of 1 µg. Based on these results, the authors indicated that these compounds were the semiochemicals attracting the parasite into the cell. However, successive studies did not confirm the biological activity of such chemicals (Rosenkranz, 1993; Zetlmeisl and Rosenkranz, 1994; Boot,

* Correspondence and reprints
E-mail: francesco.nazzi@pldef.uniud.it

1994). Rickli et al. (1992) demonstrated that palmitic acid, which was detected among the volatiles emitted by bee larvae, attracted the mite on a servosphere. Later Rickli et al. (1994) showed that some saturated, straight chain, odd-numbered C19–C29 hydrocarbons found in cuticle extracts of 8-day-old larvae induced an arrestment response in *V. destructor*. Arrestment is also caused by aliphatic alcohols and aldehydes of the honey bee cocoon (Donzé et al., 1998). Aumeier and Rosenkranz (1995) reported that some straight chain saturated and monounsaturated C21 to C35 hydrocarbons found in pentane extracts of *l5* bee larvae were attractive to the mite in a bioassay.

However, Kraus (1993) and LeDoux et al. (2000) noted that when offered a choice between bee larvae from nearly capped brood cells and adult nurse bees, *V. destructor* prefers the latter. As the mite is carried to the brood cell by a nurse bee, the mite's preference for nurse bees over bee larvae suggests that the odour of the brood itself may not be the most important factor involved in the process of entrance into the cell. Furthermore, most of the active compounds identified so far in bee larval extracts are widespread inside the hive. Several saturated and unsaturated hydrocarbons, whose biological activity on *V. destructor* has bee demonstrated so far, were identified both in beeswax (Tulloch, 1980) and the cuticle of adult bees (Francis et al., 1985); and some of them have been identified also on the cuticle of the mite itself (Nation et al., 1992). Palmitic acid was identified in the cuticle of adult bees (Blomquist et al., 1980), honey (Crane, 1979), pollen (Bee World, 1975) and propolis (Marcucci, 1995). Such products may be involved in the process of host recognition but may not be the major signals triggering the entrance of the mite into the brood cell.

At the time the mite enters a drone brood cell, it contains about 20 milligrams of larval food, and a smaller quantity of food is present in worker cells. The mite often gets trapped in the larval food from which it leaves 2 to 6 hours after cell capping (Ifantidis, 1988). Previous studies indicated that larval food can influence mite reproduction (Milani and Chiesa, 1991) suggesting the presence of semiochemicals that influence the behaviour and/or the physiology of the parasite. A statement about the attractivity of larval food to *V. destructor* exists in a little known paper (Issa et al., 1985).

The aim of this research was to investigate the role of larval food in the process of mite entrance into the brood cell.

2. MATERIALS AND METHODS

2.1. Biological material

Bee larvae and mites used in all the experiments came from untreated, local *Apis mellifera* L. colonies maintained in Udine (northeastern Italy). The mites and bee larvae were obtained from brood cells 0–15 hours postcapping (*0–15 PC*) according to Chiesa et al. (1989). Fifth instar bee larvae before capping (*l5 BC*) were manually extracted from unsealed bee brood cells. Larval food was extracted with a small spatula from drone cells containing either a fourth or fifth instar larva and kept at –20 °C in sealed vials until used.

2.2. Bioassay

An arena similar to that used by Rosenkranz (1993) was used for the bioassays. It consisted of a glass plate with four wells (7 mm diameter; 8 mm deep) equidistant (1 cm) from the centre. A glass lid mounted on a circular metal ring (5.6 cm diameter) was used to confine the mites in the arena. A treatment was applied to two opposite wells while the other two wells were used as controls. Then one bee larva was placed into each well. At the beginning of the bioassay one adult female mite was

placed with a fine paint brush in the centre of the arena between the four wells. Arenas were kept in an environmental chamber at 35 °C and 75% R.H. for the duration of the bioassay. The chamber was opened and the position of the mites noted every 5 minutes (except in experiment b), and bioassays lasted 30 minutes. Twenty arenas were used at a time. Tests were replicated on different days.

2.3. Experiments with biological substrates

2.3.1. Response to larvae of different ages

The first set of bioassays was designed to check the efficiency of the arena test and to select the best age of larvae to be used in the bioassay. Attraction of *V. destructor* to bee larvae of two different ages was studied by placing a *l5 BC* bee larva in each of two opposite wells and a *0–15 PC* larva in the others. The experiment was replicated 6 times using a total of 120 mites.

2.3.2. Response to methyl palmitate

One microgram of methyl palmitate dissolved in ten microliters of hexane was used as the treatment in two opposite wells; the other wells received ten microliters of hexane as a control. *0–15 PC* larvae were then placed in all wells. The experiment was replicated 3 times using a total of 60 mites.

In this set of bioassays the position of the mites in the arena was noted every 15 minutes instead of at 5 minutes intervals.

2.3.3. Response to larval food

Ten milligrams of larval food were smeared with a small spatula inside two opposite wells; control wells were left untreated. *0–15 PC* larvae were then placed in all wells. The experiment was replicated 5 times using a total of 100 varroa mites.

2.3.4. Response to semiochemicals extracted from larval food

For extraction, 500 mg of larval food was smeared using a spatula inside a conical test tube and 3 ml of solvent was added. Diethyl ether and acetone solvents were tested for their efficiency in extracting the active compounds from larval food. After 1 hour for acetone, and 24 hours for ether, the tube was centrifuged until the larval food residue was sedimented. The supernatant was transferred to another tube and was reduced to 500 µl under nitrogen. Ten µl of this extract, corresponding to 10 mg equivalents of larval food, was applied in each treated well and 10 µl of solvent was applied to the control wells. 0–15 PC larvae were then placed in the wells. The acetone extract was dried over magnesium sulfate before using. The experiment with the ether extract was replicated 8 times using a total of 160 mites, and the one with acetone extract was replicated 4 times using a total of 80 mites.

2.4. Statistical analysis of data

For each arena, the number of times the mite was observed in the treated and control wells, respectively, over the 30 minutes period were used as scores for the statistical analysis regardless of whether the mite had changed wells between observations or just stayed in the same wells. Then, a matrix was constructed with as many rows as the number of mites used in the bioassay, and two columns containing the scores for treated and control wells for each of the tested mites. The treated and control scores in a given set of data were compared by a sampled randomization test (Manly, 1991; Sokal and Rohlf, 1995). The randomization distribution was resampled 10^6 times with a computer programme written for this purpose. A sampled randomization test was chosen because the distribution of the variables to be compared was unknown; in this case conventional parametric statistics often lead to an overestimate of the significance of differences.

3. RESULTS

3.1. Response to larvae of different ages

The difference between scores in wells containing larvae from unsealed cells (*15 BC*) and scores in wells containing larvae from sealed cells (*0–15 PC*) was significant ($P = 0.016$; Tab. I).

3.2. Response to methyl palmitate

No significant difference was found between scores in treated and control wells when methyl palmitate was used as a treatment (sum of scores in treated wells = 38, sum of scores in control wells = 33; $P = 0.379$).

3.3. Response to larval food

When larval food was applied to treated wells the difference between scores in treated and control wells was highly significant ($P < 0.001$; Tab. II).

3.4. Response to semiochemicals extracted from larval food

Both the ether and the acetone extract of larval food elicited a significant response from *V. destructor*, in that the difference between scores in treated and control wells was highly significant ($P < 0.01$ in both cases; Tabs. III and IV).

4. DISCUSSION

During the bioassay *V. destructor* can move freely within the arena, entering and leaving different wells, such that the number of times each mite was observed inside the treated and control wells at the end of the bioassay is related both to the number of entries into the wells and the time spent

Table I. Response of *V. destructor* to larvae of different ages. Sum of scores of 20 mites, each observed every 5 min over 30 min, in wells containing a fifth instar bee larva before capping (*l5 BC*) or a larva from a sealed cell (*0–15 PC*).

Replication	*l5 BC*	*0–15 PC*	*P*
1	33	8	0.027
2	36	36	0.521
3	37	32	0.418
4	40	16	0.066
5	33	12	0.067
6	32	24	0.333
Tot.	211	128	0.016

Table II. Response of *V. destructor* to larval food. Sum of scores of 20 mites, each observed every 5 min over 30 min, in wells treated with 10 mg of larval food (treated) or untreated (control).

Replication	Treated	Control	*P*
1	71	11	0.000
2	53	11	0.008
3	35	4	0.010
4	54	9	0.001
5	36	19	0.121
Tot.	249	54	0.000

Table III. Response of *V. destructor* to an ether extract of larval food. Sum of scores of 20 mites, each observed every 5 min over 30 min, in wells treated with 10 mg equivalent of an ether extract of larval food (treated) or 10 µl of ether (control).

Replication	Treated	Control	*P*
1	21	1	0.004
2	19	3	0.012
3	19	8	0.094
4	5	0	0.125
5	17	15	0.465
6	39	13	0.030
7	15	9	0.289
8	17	7	0.143
Tot.	152	56	0.000

Table IV. Response of *V. destructor* to an acetone extract of larval food. Sum of scores of 20 mites, each observed every 5 min over 30 min, in wells treated with 10 mg equivalent of an acetone extract of larval food (treated) or 10 µl of acetone (control).

Replication	Treated	Control	*P*
1	36	9	0.005
2	33	0	0.000
3	19	7	0.055
4	22	6	0.040
Tot.	110	22	0.000

inside them. For this reason the bioassay used in this study does not make it possible to discriminate between the attractivity or the arrestment effect of the stimulus under test but can indicate whether this stimulus can be involved in the process of cell invasion by the mite. In fact, under natural conditions, the entrance into the cell by the mite is probably the result of a combination of attraction to the brood and arrestment inside the cell.

The response of *V. destructor* to fifth instar bee larvae before capping was stronger than to larvae from sealed cells. The result of this experiment confirmed the efficiency of the bioassay and prompted the choice of the less active *0–15 PC* larvae in the assays designed to test the biological activity of non-larval stimuli. The higher response to the *15 BC* larvae could be due to compounds present on the cuticle such as hydrocarbons (Rickli et al., 1994; Aumeier and Rosenkranz, 1995). On the other hand, some active compounds could also be present on the larval cuticle arising from contamination by substances from the cell such as larval food.

No response to methyl palmitate was found in this study, which confirms previous findings that this ester, although important in triggering cell capping by bees (Le Conte et al., 1990), is probably not involved in the attraction of the mite to the brood cell (Rosenkranz, 1993; Zetlmeisl and Rosenkranz, 1994; Boot, 1994).

A clear response of *V. destructor* to larval food was observed. The biological activity of the extracts of larval food demonstrates that the observed effect was due to semiochemicals contained in the larval food itself. The results suggest that cues coming from a source different from the host itself could be involved in the process of cell invasion by the mite, although a more definitive conclusion can be drawn only after the activity of such stimuli under natural conditions is demonstrated.

Larval food is present inside the brood cell throughout the development of the honeybee larva. Temporal variation of the chemical composition of larval jelly (Lercker et al., 1994) as well as other factors, such as the increasing amount of jelly and the decreasing distance between larva and cell rim (Boot et al., 1995), could explain why the invasion of the cell occurs only a short time before cell capping. The different attractiveness of worker and drone cells (Fuchs, 1990) could depend on the greater quantity of larval food contained in drone cells, or possible differences in the composition of the larval food provided to female and male brood.

The identification of the substances responsible for the biological activity of larval food could contribute to a better understanding of the biology of the mite and suggest novel methods to control the parasite.

AKNOWLEDGEMENTS

This research was carried out in the frame of the AMA project ("Ape, Miele e Ambiente"), financed by the Italian "Ministero delle Politiche Agricole e Forestali", paper No. 84.

Résumé – Des médiateurs chimiques provenant de la nourriture larvaire affectent le comportement locomoteur de *Varroa destructor*. Divers composés qui attirent ou

arrêtent *Varroa destructor* ont été identifiés, mais les signaux qui déclenchent l'invasion des cellules du couvain par l'acarien n'ont pas encore été identifiées. Lorsque l'acarien pénètre dans une cellule de couvain, celle-ci renferme une quantité variable de nourriture larvaire dont on a suggéré qu'elle exerçait une activité biologique sur le parasite. Nous avons étudié au laboratoire, à l'aide d'un test biologique, le rôle de la nourriture larvaire dans le processus d'invasion des cellules : un acarien a été placé au centre d'une arène en verre munie de 4 puits renfermant chacun une larve d'abeille prélevée dans une cellule operculée depuis 0–15 h. Deux puits ont été traités avec la substance à tester, les deux autres ont servi de témoins. Le comportement de l'acarien dans l'arène a été observé toutes les 5 min durant 30 min.

Les larves traitées à la nourriture larvaire ont été nettement plus visitées par les acariens que les non traitées (Tab. II). Un extrait à l'éther et un extrait à l'acétone de la nourriture larvaire se sont montrés tous deux actifs au cours du test biologique (Tabs. III et IV), ce qui suggère un rôle possible des médiateurs chimiques provenant de la nourriture larvaire dans le processus d'invasion des cellules de couvain par le parasite.

***Varroa destructor* / médiateur chimique / nourriture larvaire / invasion des cellules**

Zusammenfassung – Semiochemische Substanzen aus der Larvennahrung beeinflussen die Fortbewegung von *Varroa destructor*. Es sind verschiedene Komponenten mit attraktiver oder bewegungshemmender Wirkung auf *Varroa* bekannt. Die Reize, welche die Einwanderung der Milben in die Brutzellen auslösen, wurden bislang allerdings nicht identifiziert. Zu dem Zeitpunkt, an dem *Varroa* die Brutzellen befällt, enthalten diese eine unterschiedliche Menge von Larvennahrung. Nach früheren Vermutungen könnte diese für die Milben biologisch aktiv sein. Wir untersuchten die mögliche Rolle der Larvennahrung in dem Prozess der Zellinvasion im Labor unter Verwendung eines Biotests: Eine Milbe wurde in eine Glasarena in die Mitte von vier Vertiefungen gesetzt, von denen jede eine Larve aus einer 0–15 Stunden verdeckelten Zelle enthielt. Zwei der Vertiefungen wurden mit der zu untersuchenden Substanz behandelt, die anderen beiden dienten als Kontrolle. Das Verhalten der Milbe wurde während 30 Minuten alle 5 min protokolliert.

Mit Larvennahrung behandelte Larven waren im Biotest wesentlich stärker von den Milben besucht (Tab. II). Sowohl ein Etherextrakt als auch ein Acetonextrakt der Larvennahrung zeigten in dem Biotest ebenfalls eine deutliche Wirkung (Tab. III und IV). Die Ergebnisse weisen auf eine mögliche Rolle von semiochemische Substanzen aus der Larvennahrung in dem Prozess der Zellinvasion hin.

***Varroa destructor* / Semiochemische Substanzen / Larvennahrung / Brutzelleninvasion**

REFERENCES

Aumeier P., Rosenkranz P. (1995) Welche Faktoren der Bienenlarven kutikula beeinflussen die Wirtsfindung der Varroa-Weibchen, Apidologie 26, 327–329.

Bee World (1975) What we know about pollen, Bee World 56, 155–158.

Blomquist G.J., Chu A.J., Remaley S. (1980) Biosynthesis of wax in the honeybee, *Apis mellifera* L., Insect Biochem. 10, 313–321.

Boot W.J. (1994) Methyl palmitate does not elicit invasion of honeybee brood cells by Varroa mites, Exp. Appl. Acarol. 18, 587–592.

Boot W.J., Calis J.N.M., Beetsma J. (1992) Differential period of *Varroa* mite invasion into worker and drone cells of honey bees, Exp. Appl. Acarol. 16, 295–301.

Boot W.J., Driessen R.G., Calis J.N.M., Beetsma J. (1995) Further observations on the correlation between attractiveness of honeybee brood cells to *Varroa jacobsoni* and the distance from larva to cell rim, Entomol. Exp. Appl. 76, 223–232.

Chiesa F., Milani N., D'Agaro M. (1989) Observations of the reproductive behaviour of *Varroa jacobsoni* Oud.: techniques and preliminary results, in: Cavalloro R. (Ed.), Present status of varroatosis in Europe and progress in the varroa mite control. Proc. Meeting EC-Experts Group, Udine 1988, C.E.C., Luxembourg, pp. 213–222.

Crane E. (1979) Honey. A comprehensive survey, Heinemann, London.

Donzé G., Schnyder-Candrian S., Bogdanov S., Diehl P.A., Guerin P.M., Kilchenman V., Monachon F. (1998) Aliphatic alcohols and aldehydes of the honey bee cocoon induce arrestment behavior in *Varroa jacobsoni* (Acari: Mesostigmata), an ectoparasite of *Apis mellifera*, Arch. Insect Biochem. 37, 129–145.

Francis B.R., Blanton W.E., Nunamaker R.A. (1985) Extractable surface hydrocarbons of workers and drones of the genus *Apis*, J. Apic. Res. 24, 13–26.

Fuchs S. (1990) Preference for drone brood cells by *Varroa jacobsoni* Oud. in colonies of *Apis mellifera carnica*, Apidologie 21, 193–199.

Ifantidis M.D. (1988) Some aspects of the process of *Varroa jacobsoni* mite entrance into honey bee (*Apis mellifera*) brood cells, Apidologie 19, 387–396.

Issa M.R.C., De Jong D., Gonçalves L.S. (1985) Étude sur la préférence de l'acarien *Varroa jacobsoni* pour les faux bourdons d'*Apis mellifera*, Proc. XXXth Congr. Apiculture., Nagoya, 1985, Apimondia Publ. House, Bucharest, pp. 168–170.

Kraus B. (1993) Preferences of *Varroa jacobsoni* for honey bees (*Apis mellifera* L.) of different ages, J. Apic. Res. 32, 57–64.

Le Conte Y., Arnold G., Trouiller J., Masson C., Chappe B., Ourisson G. (1989) Attraction of the parasitic mite *Varroa jacobsoni* to the drone larvae of honeybees by simple aliphatic esters, Science 245, 638–639.

Le Conte Y., Arnold G., Trouiller J., Masson C., Chappe B. (1990) Identification of a brood pheromone in honeybees, Naturwissenschaften 77, 334–336.

LeDoux M.N., Pernal S.F., Higo H.A., Winston M.L. (2000) Development of a bioassay to test the orientation behaviour of the honey bee ectoparasite *Varroa jacobsoni*, J. Apic. Res. 39, 47–54.

Lercker G., Vecchi M.A., Piana L., Nanetti A., Sabatini A.G. (1994) Composition de la fraction lipidique de la gelée de larves d'abeilles reines et ouvrières (*Apis mellifera ligustica* Spinola) en fonction de l'âge des larves, Apidologie 15, 303–314.

Manly B.F.J. (1997) Randomization, bootstrap and Monte Carlo methods in biology, Chapman and Hall, London.

Marcucci M.C. (1995) Propolis: chemical composition, biological properties and therapeutic activity, Apidologie 26, 83–99.

Milani N., Chiesa F. (1991) Some stimuli inducing oviposition in *Varroa jacobsoni* Oud, Ritter W. (Ed.), Proc. Int. Symp. Recent research on bee pathology, Gent 1990, Apimondia, Bucharest, pp. 27–33.

Nation J.L., Sanford M.T., Milne K. (1992) Cuticular hydrocarbons from *Varroa jacobsoni*, Exp. Appl. Acarol. 16, 331–344.

Rickli M., Guerin P.M., Diehl P.A. (1992) Palmitic acid released from honeybeee worker larvae attracts the parasitic mite *Varroa jacobsoni* on a servosphere, Naturwissenschaften 79, 320–322.

Rickli M., Diehl P.A., Guerin P.M. (1994) Cuticle alkanes of honeybee larvae mediate arrestment of bee parasite *Varroa jacobsoni*, J. Chem. Ecol. 20, 2437–2453.

Rosenkranz P. (1993) Biotest zur Untersuchungen des Wirtsfindenverhaltens von *Varroa jacobsoni*, Apidologie 24, 486–488.

Sokal R.R., Rohlf F.J. (1995) Biometry: The Principles and Practice of Statistics in Biological Research, Freeman and Co., New York, 3rd ed.

Tulloch A.P. (1980) Beeswax – composition and analysis, Bee World 61, 47–62.

Zetlmeisl K., Rosenkranz P. (1994) Varroa-Weibchen im Biotest: Wirtserkennung von Bienenlarven und adulten Bienen, Apidologie 25, 507–508.