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Original article

Stimulation of *Varroa jacobsoni* Oud. oviposition with semiochemicals from honeybee brood

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Abstract – The oviposition of female *Varroa jacobsoni* kept with worker larvae of honeybees (*Apis mellifera*) in artificial gelatine cells was stimulated when 5-larva equivalents of extracts of L5 worker larvae were applied on the inner side of the walls of the cells. Most of the stimulating activity was recovered in the most polar fraction of the larval extract. The corresponding fraction of an extract of pupae did not stimulate oviposition. Semiochemicals present on the surface of L5 worker larvae, but not on pupae, may play a role in the regulation of *V. jacobsoni* reproduction. © Inra/DIB/AGIB/Elsevier, Paris

***Varroa jacobsoni* / artificial rearing / fertility / kairomone / oviposition / semiochemical**

1. INTRODUCTION

The parasitic mite *Varroa jacobsoni* Oudemans (Acari, Varroidae) reproduces exclusively on the brood of *Apis mellifera* L., *A. cerana* Fabricius and some other Asian *Apis* species. In *A. mellifera*, a variable proportion of *V. jacobsoni* females does not lay eggs, although they may be fertile in other reproductive cycles [6, 22]. The fertility is usually higher on drone brood than on worker brood. In *A. cerana*, the *V. jacobsoni* females reproduce almost only on drone brood [23], and the tolerance of *A. cerana* to the mite is partly due to the fact that the parasite reproduction is restricted to the period when drone brood is present. The fertility rate of *V. jacobsoni* females infesting Africanized honeybees in Brazil is very low compared to what is generally observed with European honeybees [5, 18]. However, in Mexico, reduced fertility of the mite does

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not seem to be the main factor for Africanized bee tolerance to the mite [24]. Differences in the fertility seem to be related to either honeybee race or to the local strain of the mite [1, 4]. It has been shown that reproduction of *V. jacobsoni* is influenced by factors acting during the phoretic phase [3, 6, 9, 19, 22] and during the first contact with the bee larva inside the brood cell [9, 13, 21]. In both cases, the factors acting on the mite are unknown.

When the *V. jacobsoni* female enters into contact with the bee larva, just after capping, the oocytes are at the previtellogenic stage. The embryogenesis starts only 30 h after the cell has been capped and only if the mite has been in contact with an L5 larva 0–24 h after capping [7, 21, 22]. A first oocyte matures into an egg, laid 60–70 h after capping [8, 11, 14]. It seems that the 30-h interval following capping is one of the key periods for the regulation of reproduction. It has been shown, under experimental conditions, that the mite could start oviposition only if it had previously entered into contact with a bee larva aged 0–48 h after capping [2, 10, 15]. However, this does not explain what are the factors leading to oviposition. A better knowledge of the reproduction process could clarify some aspects of bee tolerance and, with the help of chemical markers, be used to select strains of tolerant bees.

The objectives of this study were to determine if oviposition of *V. jacobsoni* was stimulated by semiochemicals present on worker honeybee larvae during the L5 stage, but not during pupal stage, and to isolate these potential semiochemicals.

2. MATERIAL AND METHODS

Experiments were carried out between 1995 and 1997 at the University of Udine (Italy) on mites and bees belonging to the local populations. Bees were free outdoor hybrids between *A. m. ligustica* Spinola and *A. m. carnica* Pollman. The influence of honeybee brood extracts on *V. jacobsoni* reproduction was tested under laboratory conditions. For all experiments, the mites and bee larvae were alternatively taken from one of the five honeybee colonies placed in a single isolated apiary. These colonies had not been treated for varroatosis since 1993.

2.1. Worker larvae and pupae extract

In a colony, frames containing open brood were selected and the capped cells were marked on a transparency applied on the frames. Twelve hours later, newly capped cells were opened and the frames were placed in an incubator. For every step of the experiments, the incubators were at 34 ± 1 °C and 75 ± 5 % RH. To avoid possible damage using forceps, the worker larvae were allowed to fall freely from their cells for 2 h. For the pupal extract, a frame was taken from a colony and the caps were opened. Pupae (white to pale brown body, 5–10 days after capping) were removed with a forceps from the cells.

Cuticular compounds of such larvae and pupae were extracted with pentane (0.5 mL/larva or pupa). The extracts were concentrated under a flow of nitrogen until a final concentration of 5-larva or -pupa equivalents/25 µL.

2.2. Fractions of brood extracts

The larvae and pupae extracts were fractionated on a custom-made silica column (2 g of 40–60-µm glass beads packed in a glass column, 15 cm long and 5 mm in diameter). The first fraction (F1) was obtained by passing 20 mL of pentane through the column, the F2 fraction with 20 mL of a mixture of pentane and diethyl ether (95:5 v/v), and the fraction F3 with 20 mL of diethyl ether. To avoid effects caused by the different solvents used, diethyl ether and pentane were added to F1 and F3, respectively; consequently, every fraction contained a mixture of these two solvents. The fractions were concentrated under a flow of nitrogen to a final concentration of 5-larva or pupa equivalents/25 µL.

2.3. *V. jacobsoni* reproduction test

The test used was as described by Nazzi and Milani [16, 17].

2.3.1. Rearing capsules

V. jacobsoni females were reared on worker larvae in pharmaceutical gelatine capsules (Capsugel, Colmar, France). Capsules no. 0 (7 mm in diameter and 20 mm long) were used in the experiments carried out in 1995 and 1996, and capsules no. 1 (6.5 mm in diameter and 20 mm long) in the 1997 experiments.

2.3.2. Treatments applied onto the capsules

Twenty-five microlitres of brood extract or fraction of brood extract were applied on the inner side of the walls of the capsule 1 h before the introduction of the bee larva and the *V. jacobsoni* female; controls were treated with a mixture of solvents (pentane/diethyl ether). Preliminary observations using doses of 2-, 5- and 10-larva equivalents showed that the stimulating effect of brood extract was not increased using more than 5-larva equivalents. Consequently, we used the dose of 5-larva equivalents in all the experiments. The capsules were closed with their lid in which three holes had been made with a number 2 insect pin.

2.3.3. Collection of bee larvae and mites

In an infested colony, the capped cells were marked using a transparency. Twelve hours later, the cells that had been capped during this period were opened and the frames placed in an incubator. The length of the period was chosen so as to insure having enough mites and honeybee larvae from a single colony for each replicate. The larvae were allowed to fall from the cells for 2 h; larvae and mites were collected and kept in Petri dishes in an incubator.

2.3.4. Experiments

A larva and a mite were assigned at random to the control or to one of the treated groups and inserted into a capsule, the capsules being then kept in a Petri dish placed in the incubator. In each experiment, control capsules and treated capsules were prepared at the same time and kept under the same conditions. After 8 days (experiments carried out in 1995–1996) or 4 days (1997) the capsules were opened. Their walls and the bee pupae were inspected under a dissecting microscope. The capsules containing a dead mother mite and/or a dead pupa were set aside and the number of fertile (at least one offspring alive or dead) and non-fertile mites was recorded.

2.3.5. Replications

Each experiment was replicated 2 to 5 times. A single colony was used as a source of bee larvae and mites in each replicate, but different colonies were used for the different replicates. The number of capsules used for each replicate depended on the number of mites available. Brood extracts were prepared with larvae or pupae from a single colony of the apiary, but the colony changed from one replicate to another.

2.4. Statistical analysis

The number of mites differed among replicates because variable numbers of mites were available each time and because of variation in brood and/or mite mortality (9–41 %). We compared the mortality and fertility data of each replicate of each treatment with its corresponding control. To insure that a variation in the mortality between replicates and treatments did not impair the fertility data, we tested the homogeneity of mortality for each treatment with its corresponding control. We also tested the homogeneity of fertility between the replicates and their corresponding controls. Homogeneity was tested using the Mantel-Haenszel procedure (χ^2 of adjusted log odds ratio (χ^2_{ALO}); [20]). The significance of the treatment effect on mortality and fertility was tested using the modified Mantel-Haenszel χ^2 (χ^2_{MH}). When the treatment effect was significant, the Mantel-Haenszel estimator of the odds ratio (w_{MH}) for fertility was calculated [20].

3. RESULTS

3.1. Mortality

For each type of experiment, brood and/or mite mortality (*table I*) was homogeneous between the different replicates of each treatment and their corresponding controls, except for the fraction F1 of pupal extract (larval extract 1995–1996: $\chi^2_{ALO} = 5.08$, df = 4, $P = 0.28$; larval extract 1997:

Table I. Mortality of brood and/or mites during the test according to the treatment used.

Experiment	Treatment	Total number of capsules	Mortality (%)
Larval extract	control	118	31
1995–1996	extract	112	38
Larval extract	control	124	12
1997	extract	128	9
Larval extract	control	108	31
fractions	F1	107	38
1997	F2	106	36
	F3	99	40
Pupal extract	control	87	25
fractions	F1	87	19
1997	F2	82	26
	F3	79	38

$\chi^2_{ALO} = 1.06$, df = 1, $P = 0.30$; fractions of larval and pupal extracts: $\chi^2_{ALO} < 3.15$, df = 2, $P > 0.20$). For the fraction F1 of pupal extract, the first replicate was not homogeneous with the two others ($\chi^2_{ALO} = 6.34$, df = 2, $P = 0.04$) and was characterized by a mortality significantly lower than that observed with the corresponding control (Williams adjusted G = 16.63, df = 1, $P < 0.001$). The two other replicates did not show a significant difference with the control ($\chi^2_{MH} = 0.29$, df = 1, $P = 0.59$). Overall, there was no significant difference in the mortality between control and treated capsules (for all experiments: $\chi^2_{MH} < 1.58$, df = 1, $P > 0.20$).

3.2. Worker larvae extracts

The fertility of mites kept in capsules treated with a larval extract was higher than in the control for every replicate repeated several times, and using brood and mites from different colonies and at different seasons (*tables II* and *III*). The different replicates were homogeneous within each set of experiments and were then grouped accordingly (experiment 1995–1996: $\chi^2_{ALO} = 0.66$, df = 4, $P = 0.96$; experiment 1997: $\chi^2_{ALO} = 0.59$, df = 1, $P = 0.44$; fraction experiments: $\chi^2_{ALO} < 4.47$, df = 2, $P > 0.10$). The application of 5-larva equivalents on capsule walls showed a significant stimulating effect on *V. jacobsoni* fertility (1995–1996: $\chi^2_{MH} = 7.61$, df = 1, $P = 0.006$; 1997: $\chi^2_{MH} = 5.17$, df = 1, $P = 0.02$) and it increased the odds for fertility 2.8 and 2.0 times, respectively, for 1995–1996 and 1997.

3.3. Fractions of worker larvae and pupae extracts

The fractions 1 and 2 of larval extracts did not show any significant effect on mite fertility (F1: $\chi^2_{MH} = 0.09$, df = 1, $P = 0.76$; F2: $\chi^2_{MH} = 0.58$, df = 1, $P = 0.45$; *table IV*). The stimulating activity of larval extract was recovered mainly in the third and more polar fraction ($\chi^2_{MH} = 6.53$, df = 1, $P = 0.01$; *table V*). Compared to the control, the odds for fertility in the F3 treated capsules were increased 2.8 times. The fertility rate

Table II. Effect of 5-larva equivalents worker L5 larval extracts (E) on mite fertility compared to control (C) (1995–1996).

Replicate	Treatment	Total number of mites	Percentage of fertile mites
1	C	13	31
	E	12	58
2	C	13	15
	E	13	31
3	C	16	31
	E	9	67
4	C	20	35
	E	19	63
5	C	20	30
	E	16	44
	Treatment	Mean fertility	s.d. of fertility
	C	28.4	7.6
	E	52.5	15.0

Table III. Effect of 5-larva equivalents worker L5 larval extracts (E) on mite fertility compared to control (C) (1997).

Replicate	Treatment	Total number of mites	Percentage of fertile mites
1	C	62	21
	E	44	41
2	C	47	34
	E	63	46
	Treatment	Mean fertility	s.d. of fertility
	C	27.5	9.2
	E	43.5	3.6

obtained with the third fraction (73 %) was not far from that observed in natural conditions, which is about 85 % [14]. None of the fractions of pupal extracts showed any significant effect on mite fertility (F1: $\chi^2_{MH} = 0.10$, df = 1, $P = 0.75$; F2: $\chi^2_{MH} = 0.47$, df = 1, $P = 0.49$; F3: $\chi^2_{MH} = 0.07$, df = 1, $P = 0.79$; *table V*). Even when only the two homogeneous replicates of F1 fraction of pupal extracts only were taken into account, no significant effect was observed (F1: $\chi^2_{MH} = 0.29$, df = 1, $P = 0.59$).

4. DISCUSSION

The existence of reproduction stimulating semiochemicals which can be extracted from

Table IV. Effect of different fractions of worker L5 larval extracts on mite fertility.

Replicate	Treatment	Total number of mites	Percentage of fertile mites
1	C	26	46
	F1	29	48
	F2	31	61
	F3	22	77
2	C	28	50
	F1	20	40
	F2	21	57
	F3	19	74
3	C	21	52
	F1	17	47
	F2	16	50
	F3	18	67
	Treatment	Mean fertility	s.d. of fertility
	C	49.5	3.1
	F1	45.1	4.5
	F2	56.1	5.7
	F3	72.5	5.4

Table V. Effect of different fractions of worker pupal extracts on mite fertility.

Replicate	Treatment	Total number of mites	Percentage of fertile mites
1	C	14	71
	F1	28	39
	F2	17	47
	F3	20	70
2	C	13	46
	F1	16	44
	F2	13	46
	F3	15	53
3	C	24	58
	F1	26	73
	F2	20	55
	F3	15	67
	Treatment	Mean fertility	s.d. of fertility
	C	58.6	12.6
	F1	52.0	18.4
	F2	49.4	4.9
	F3	63.3	8.8

L5 worker larvae cuticle was demonstrated and the fraction containing at least part of these semiochemicals was isolated. These findings can explain why the contact between the mite and the bee larva shortly after capping is important for the initiation of the reproduction of the mite [15, 21].

The fertility observed in the control group was lower than that observed in natural conditions in European bee races [12, 14]. This could be a consequence of different factors such as manipulation of the mites and bee larvae, use of capsules larger than natural brood cells, and variations in the contact time with a larva in the L5 stage (the period is shorter when a mite from a larva capped just before the collection is put onto an older larva).

Part of the variation in the fertility in the control groups (15–71 %) was due to the use of capsules of different diameters, since in larger capsules the fertility remained lower [16]. However, the difference in size could not explain some of the low values found in the experiments carried out in 1997 because all the capsules used were of the same size. On the other hand, seasonal variations in the *V. jacobsoni* fertility on European bees under natural conditions are well known [12].

The odds for fertility increased more with treatment with the fraction F3 (2.8) than that observed with whole extract with the same protocol (2.0). However, it is possible that active components were present also in other fractions, since the fraction F3 was not directly compared with the crude extract.

The stimulating effect of brood extract was tested on mites which had been in contact with L5 larvae before and during the experiment. Consequently, it was not possible to define the role of semiochemicals contained in the brood extract in the initiation of the reproductive cycle of the mite; other substances from the bee larva or the rearing cells used in the experiments could also be involved in the initiation of the reproduction.

The F3 fraction at the pupal stage did not show a stimulating effect, possibly because the stimulating semiochemicals were in smaller quantities or inhibiting substances were present during this stage. These data suggest that changes in the cuticular lipids that influence the reproduction of *V. jacobsoni* take place during the development of the brood. Different concentrations of these semiochemicals for different bee species, races, strains, sexes or castes could be some of the factors that cause the host-related differences in the fertility of the *V. jacobsoni* mite.

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Résumé – Stimulation de la ponte chez *Varroa jacobsoni* par des médiateurs chimiques issus du couvain d'abeille. L'infertilité des femelles de *V. jacobsoni* est l'une des causes de la tolérance de certaines souches d'abeilles vis-à-vis de l'acarien. On sait que les premières heures de contact entre l'acarien et la larve d'abeille, dans la cellule qui vient d'être operculée, jouent un rôle décisif dans la détermination de la fertilité ou de l'infertilité de l'acarien femelle, bien que les facteurs déterminants restent eux-mêmes inconnus. Notre objectif était d'isoler les médiateurs chimiques potentiels présents sur la larve d'abeille et susceptibles d'influer sur la fertilité des acariens. L'action d'extraits de couvain d'abeille sur la reproduction de *V. jacobsoni* a été testée au laboratoire. Les acariens et les larves hôtes de 5^{e} stade (L5) ont été placés dans des capsules de gélatine enduites de divers extraits de couvain (l'équivalent de cinq larves ou nymphes) et mis en étuve. La fertilité des acariens a été contrôlée quatre jours plus

tard, dans les expériences de 1997, et huit jours plus tard, dans celles de 1995–1996. Sauf pour une répétition unique de la fraction F1 de l'extrait de nymphe, il n'y a pas eu de différence dans la mortalité du couvain et/ou de l'acarien entre les capsules traitées et les capsules témoins (*tableau I*). On ne s'attendait donc pas à ce que la mortalité modifie l'ensemble des données de la fertilité. Parmi toutes les expériences et pour tous les traitements et leur témoin correspondant, les répétitions se sont montrées homogènes et on a considéré qu'elles avaient eu lieu dans les mêmes conditions. Pour chaque traitement, les répétitions ont été groupées selon la procédure de Mantel-Haenszel. Dans les capsules de gélatine dont la paroi intérieure avait été enduite d'extrait de larve L5 d'ouvrière, la fertilité des femelles de *V. jacobsoni* a été supérieure à celles des acariens présents dans les capsules qui avaient été traitées intérieurement avec le solvant (*tableau II* : 1995–1996 : $Chi^2_{MH} = 7{,}61$, df = 1, $p < 0{,}01$; *tableau III* : $Chi^2_{MH} = 5{,}17$, df = 1, $p < 0{,}05$). Cet effet de stimulation a été retrouvé avec la fraction la plus polaire (F3) de l'extrait de larve d'ouvrière (*tableau IV* : F3 : $Chi^2_{MH} = 6{,}53$, df = 1, $p < 0{,}02$). Le traitement des capsules avec l'extrait larvaire ou avec la fraction F3 de cet extrait a augmenté les chances de fertilité de 2 à 2,8 fois par rapport au traitement témoin. On n'a pas observé d'action stimulatrice significative avec les fractions F1 et F2 (*tableau IV* : F1 : $Chi^2_{MH} = 0{,}09$, df = 1, ns, F2 : $Chi^2_{MH} = 0{,}58$, df = 1, n.s.). Aucune action stimulatrice n'a été trouvée au stade nymphal (*tableau V* ; F1 : $Chi^2_{MH} = 0{,}10$, df = 1, ns, F2 : $Chi^2_{MH} = 0{,}47$, df = 1, ns ; F3 : $Chi^2_{MH} = 0{,}07$, f = 1, ns). Ces résultats peuvent expliquer l'importance du contact entre l'acarien et la larve d'abeille peu après l'operculation dans le déclenchement de la reproduction de l'acarien. Les médiateurs chimiques présents à la surface des larves L5 d'ouvrières pourraient jouer un rôle important dans la régulation de la reproduction de *V. jacobsoni*.

***Varroa jacobsoni* / médiateur chimique / kairomone / ponte / fertilité / élevage artificiel**

Zusammenfassung – Stimulation der Eilage von *Varroa jacobsoni* Oud. mit natürlichen Wirkstoffen aus der Honigbienenbrut. Eine der Ursachen der Toleranz einiger Herkunftslinien der Honigbienen gegenüber *Varroa jacobsoni* liegt in der Unfruchtbarkeit der Milbenweibchen. Es ist bekannt, dass die ersten Stunden des Kontakts der Milbe mit der Honigbienenlarve in der frisch verdeckelten Zelle eine wichtige Rolle bei der Entscheidung über Fertilität oder Infertilität spielen, wobei die entscheidenden Ursachen noch unbekannt sind. Unser Ziel war die Isolierung natürlicher Wirkstoffe aus der Honigbiene, die möglicherweise die Fertilität der Milben beeinflussen könnten. Die Wirksamkeit von Brutextrakten auf die Reproduktion von *V. jacobsoni* wurde unter Laborbedingungen getestet. Hierzu hielten wir in einem Brutschrank Milben und L5 Larven in Gelatinekapseln, die mit verschiedenen Brutextrakten überzogenen waren (5-Larven oder Puppen Äquivalente). Die Fertilität der Milben wurde nach 4 (bei den 1997 Versuchen) oder 8 Tage (in den 1995–1996 Versuchen) überprüft. Die Brut und / oder Milbensterblichkeit waren – mit Ausnahme einer einzigen Wiederholung mit Puppenextrakt – bei den Wiederholungen und ihren entsprechenden Kontrollen in jeder der Behandlungen vergleichbar (*Tabelle I*). Es war daher nicht zu erwarten, daß die Fertilitätsdaten durch die Sterblichkeit verändern werden. Die Wiederholungen waren bei allen Versuchen und ihren entsprechenden Kontrollen homogen. Deshalb wurden für jede Behandlung die Wiederholungen nach der Mantel-Haenszel Prozedur gruppiert.
Die Fertilität der Milbenweibchen war in Gelatinekapseln, deren Innenwände mit Extrakten von L5 Arbeiterinnenlarven überzogen waren, größer als in nur mit Lösungsmittel behandelten Kapseln. (*Tabelle II*:

1995–1996: $Chi^2_{MH} = 7,61$, df = 1, $P < 0,01$; *Tabelle III*: $Chi^2_{MH} = 5,17$, df = 1, $P < 0,05$). Diese stimulierende Wirkung wurde in den meisten polaren Fraktionen (F3) der Arbeiterinnenlarven gefunden (*Tabelle IV:* F3: $Chi^2_{MH} = 6,53$, df = 1, $P < 0,02$). Die Behandlung der Kapseln mit Larvalextrakt oder der Fraktion F3 dieses Extraktes erhöhte die Unterschiedlichkeit der Fertilität auf das 2 bis 2,8 fache im Vergleich zu den Kontrollen. Bei den Fraktionen F1 und F2 wurde kein stimulierender Effekt beobachtet (*Tabelle IV*: F1: $Chi^2_{MH} = 0,09$, df = 1, ns, F2: $Chi^2_{MH} = 0,58$, df = 1, ns). Extrakte der Puppenphase hatten keine stimulierende Wirkung (*Tabelle V*; F1: $Chi^2_{MH} = 0,10$, df = 1, ns, F2: $Chi^2_{MH} = 0,47$, df = 1, ns; F3: $Chi^2_{MH} = 0,07$, df = 1, ns). Diese Befunde könnten die Bedeutung des Kontaktes zwischen Milbe und Bienenlarve kurz nach der Zellverdeckelung für die Auslösung der Milbenreproduktion erklären. Auf der Oberfläche von L5 Arbeiterinnen, aber nicht von Puppenstadien vorkommende Wirkstoffe könnten daher eine bedeutende Rolle bei der Regulation der Reproduktion von *Varroa jacobsoni* spielen.

***Varroa jacobsoni* / künstliche Aufzucht / Fruchtbarkeit / Kairomone / Eilage / natürliche Wirkstoffe**

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