Journal of Invertebrate Pathology 113 (2013) 56–62

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Activation and interruption of the reproduction of *Varroa destructor* is triggered by host signals (*Apis mellifera*)

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ARTICLE INFO

Article history:
Received 26 October 2012
Accepted 14 January 2013
Available online 30 January 2013

Keywords:
Varroa destructor
Honey bee
Reproduction
Host signals
GC–MS analysis

ABSTRACT

The reproductive cycle of the parasitic mite *Varroa destructor* is closely linked to the development of the honey bee host larvae. Using a within colony approach we introduced phoretic *Varroa* females into brood cells of different age in order to analyze the capacity of certain stages of the honey bee larva to either activate or interrupt the reproduction of *Varroa* females. Only larvae within 18 h (worker) and 36 h (drones), respectively, after cell capping were able to stimulate the mite's oogenesis. Therewith we could specify for the first time the short time window where honey bee larvae provide the signals for the activation of the *Varroa* reproduction. Stage specific volatiles of the larval cuticle are at least part of these activation signals. This is confirmed by the successful stimulation of presumably non-reproducing mites to oviposition by the application of a larval extract into the sealed brood cells. According to preliminary quantitative GC–MS analysis we suggest certain fatty acid ethyl esters as candidate compounds.

If *Varroa* females that have just started with egg formation are transferred to brood cells containing host larvae of an elder stage two-thirds of these mites stopped their oogenesis. This confirms the presence of an additional signal in the host larvae allowing the reproducing mites to adjust their own reproductive cycle to the ontogenetic development of the host. From an adaptive point of view that sort of a stop signal enables the female mite to save resources for a next reproductive cycle if the own egg development is not sufficiently synchronized with the development of the host.

The results presented here offer the opportunity to analyze exactly those host stages that have the capacity to activate or interrupt the *Varroa* reproduction in order to identify the crucial host signals.

1. Introduction

The reproduction of the honey bee mite *Varroa destructor* is a crucial point for the population dynamic of this parasite (Fries et al., 1994; Calis et al., 1999). The life cycle of the female mites is subdivided into a phoretic phase on adult bees and a reproductive phase within worker or drone brood cells. For reproduction, the female mite leaves the adult bee and enters a brood cell with 5th instar larva shortly before the cell sealing and become stuck in the larval food at the bottom of the brood cell. Within a few hours after cell capping the larvae consume the rest of the food and set the mite free (reviewed in Rosenkranz et al. (2010)). At that time the female mite has already started with oogenesis in the terminal oocyte (Steiner et al., 1994; Garrido et al., 2000). In laboratory bioassays we have demonstrated that the first step of the activation of the mite's oogenesis is triggered by volatiles of the larval cuticle independently from the uptake of hemolymph by the mite (Garrido and Rosenkranz, 2004). The activating components are apparently in the polar fraction of the cuticular volatiles (Trouiller and Milani, 1999; Garrido and Rosenkranz, 2004). Additionally, stage specific factors of the host larvae and pupae, respectively, influence the sex of the mite's eggs (Garrido and Rosenkranz, 2003). Obviously, the activation as well as the course of the reproduction of *V. destructor* is closely correlated with the preimaginal development of the honey bee host. This may be an adaptation to the relatively short capping period of the honey bee worker brood. The female mite lays the first male egg approximately 70 h after cell capping followed by 3–5 female eggs in 30 h intervals (Martin, 1994; Rehm and Ritter, 1989). As the success of a reproductive cycle depends on the number of viable adult mated daughter mites that leave the brood cell together with the hatching young bee, the duration of the postcapping period is a limiting factor and, therefore, the mother mite should start egg laying as soon as possible (Rosenkranz et al., 2010).

However, the dependence of the mite reproduction from specific factors of the honey bee larvae offers possibilities for a host

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http://dx.doi.org/10.1016/j.jip.2013.01.007

adaptation in order to limit the reproductive success of the parasite. So it is a long known phenomenon that a reasonable percentage of female mites do not reproduce successfully after invading a brood cell. Some mites do not lay eggs at all (reviewed in Rosenkranz et al. (2010), Carneiro et al. (2007), Correa-Marques et al. (2003) and Garrido and Rosenkranz (2003)), others do lay male or female eggs only or show delayed egg laying (Donzé et al., 1996; Martin et al., 1997; Locke and Fries, 2011). It is yet unknown to what degree host factors are responsible for those disorders in mite reproduction.

In our approach we focused on the phenomenon "non-reproduction" in *V. destructor*. At first, we proved in detail which larval instars are capable to induce the reproduction in *Varroa* females. Using a new within-colony bioassay we tried to verify if larval volatiles can activate the reproduction in those *V. destructor* females which actually are considered infertile. In a further approach we examined whether an already started oogenesis of *Varroa* females can be interrupted by the signals of certain host stages. Finally, we analyzed the pattern of cuticular compounds of those larval stages that have an influence on the fertility of the mite.

These experiments were performed to specify the role of stage specific signals of the host larvae for initiation and disruption of *Varroa* reproduction.

2. Materials and methods

2.1. Reproduction of V. destructor after natural infestation of brood cells

We used *A. mellifera carnica* colonies from the apiary at the University of Hohenheim headed by queens of our local breeding line. From June to September brood cells of drone and worker brood combs were individually analyzed for infestation with *V. destructor*. Only single infested brood cells containing 8–9 day old pupae with dark eyes and yellow thorax (Martin, 1994) were used for further analysis. Within those brood cells the female mites have already terminated egg laying but the first daughter mite has not finished the adult molt and can therefore easily be distinguished from the mother mite. All mites that have laid at least one egg were considered "reproductive", the mites without egg laying were considered "non-reproductive".

2.2. Artificial infestation of V. destructor into brood cells of different age

To determine the exact relation between larval age and activation of the *Varroa* reproduction, worker and drone brood cells were marked shortly before sealing on transparency sheets (Aumeier and Rosenkranz, 2001). Two to six hours later, capped brood cells were marked and considered "freshly capped". These marked brood cells were used for artificial infestation with phoretic *V. destructor* mites (Garrido and Rosenkranz, 2004). Mite-free colonies were chosen as host colony for these experiments to prevent the transfer of mites to brood cells that are already infested.

Phoretic mites for the infestation of the brood cells were sampled randomly from hive bees of heavily infested colonies but without clinical symptoms of Varroosis (Rosenkranz et al., 2010). Within 1 h the mites were introduced into the chosen brood cells. For that purpose, the cell capping was carefully folded out with a razor blade and the mite was introduced with an insect pin. Then the cell capping was closed again. Phoretic mites were introduced into freshly sealed brood cells and also into brood cells 6, 12, 18, 24 and 30 h after cell capping (worker brood) and 12, 24, 36, 48 and 60 h after cell capping (drone brood), respectively. The position of the treated brood cells on the combs was marked on transparency sheets. After artificial infestation all test combs were returned to the colony in order to guarantee the required temperature and humidity inside the brood cells. The treated combs remained in the colony for the entire duration of the experiment, because *V. destructor* mites introduced into brood cells and then kept in an incubator have lower reproduction rate compared to mites kept in the colony (Ibrahim and Spivak, 2006).

Eight days later the artificially infested brood cells were analyzed for reproduction of the introduced *V. destructor* female. Again, a *Varroa* mite was considered "reproductive" when at least one offspring (e.g. egg and/or nymphal stages) were present within the brood cell. Dead mites (in total <5% of the introduced mites) were not considered in the analysis.

2.3. Reproduction of Varroa mites that have been transferred into brood cells of different ages after initiation of oogenesis

Phoretic *V. destructor* mites were introduced into freshly capped worker brood cells (see Section 2.2 for details). 24 h after introduction into brood cells, one portion of these mites were transferred to cells of the same larval age (control, Fig. 1I) or to brood cells containing larvae that were 24 h (i.e. 48 h after cell capping, Fig. 1II) and 48 h (i.e. 72 h after cell capping, Fig. 1IV), respectively, older. Another portion of mites were transferred 48 h after introduction into freshly sealed brood cells to brood cells containing larvae that were 24 h older (i.e. 72 h after cell capping, Fig. 1III).

Only female mites which had already started with oogenesis were transferred. The successful start of the oogenesis can easily and clearly be defined by the swollen idiosoma of the introduced mother mite caused by the growth of the first oocyte (Martin, 1994; Garrido and Rosenkranz, 2003; Steiner et al., 1995). The infested cells were examined 8 days after cell capping. All reproductive stages, i.e. eggs, protonymphs, deutonymphs and males were recorded.

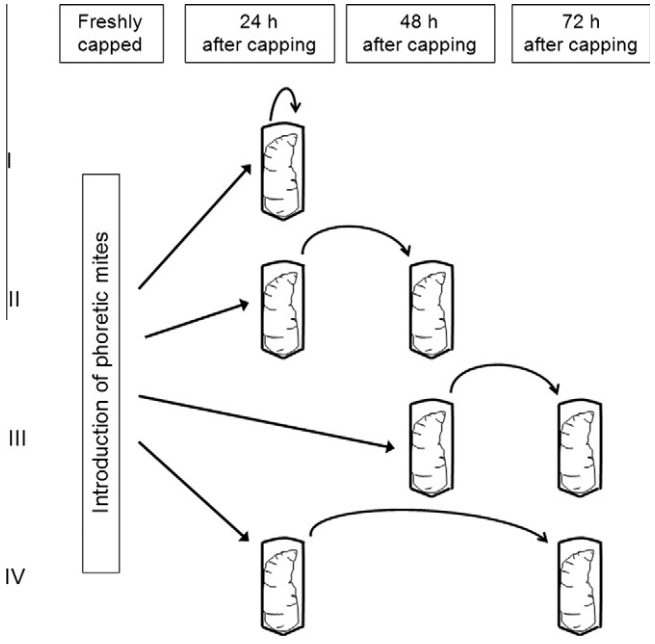

Fig. 1. Procedure of mite transfer among brood cells: phoretic mites were introduced into freshly capped brood cells and transferred after 24 and 48 h, respectively, into brood cells of different age. I (control, $n = 30$): Transfer from 24 h sealed brood cells to 24 h sealed brood cells. II ($n = 36$): Transfer from 24 h sealed brood cells to 48 h sealed brood cells. III ($n = 36$): Transfer from 48 h sealed brood cells to 72 h sealed brood cells. IV ($n = 29$): Transfer from 24 h sealed brood cells to 72 h sealed brood cells.

A subset of non-reproducing mites ($n = 36$) was dissected in order to prove the presence of spermatozoa in the spermatheca. The mites were dissected in PBS saline buffer by removing the dorsal shield to expose reproductive organs.

2.4. Application of 5th instar larval extracts into worker brood cells

The extraction was performed according to the method described by Garrido and Rosenkranz (2004). Briefly, non-infested and freshly capped worker larvae (5th instar, 0 h, see Section 2.2) were carefully removed from the brood cells. The larvae were first placed on a filter paper to check if hemolymph was leaking out and only non-injured larvae were used for the extraction. Groups of 10 larvae were pooled within a clean Erlenmeyer flask, filled up with 5 ml *n*-pentane (Uvasol) and extracted for 10 min at room temperature. Then the extract was removed from the larvae and concentrated with nitrogen to about 1 ml and stored at −20 °C. Before the start of the experiment the extract was further concentrated to 4 µl in order to reduce the amount of solvent for the application in the bioassay. These 4 µl represent the extract of ten larvae and, therefore, an application of 1 µl equals 2.5 larval equivalents.

To test the effect of these extracts on mite reproduction, we used marked worker brood cells which had already been sealed for 24 h (see above). This larval stage is not able to activate mite oogenesis (see results in Section 3.1, Fig. 2). We introduced phoretic *V. destructor* females into these brood cells. Before the introduction of the mites, we applied 2 µl of the larval extract (=5 larval equivalent) topically on the larvae in the opened brood cell ($n = 24$). To the control cells, the same amount of solvent (pentane) was applied ($n = 26$). After 2 min of evaporation of the solvent, the mite was introduced and the capping of the brood cell was closed. A third portion of the brood cells remained untreated ($n = 30$) before the introduction of the mites. All applications and controls were performed within the same colony during the same time period.

2.5. Chemical analyses

Here we focus on the quantification of cuticular methyl and ethyl esters because for these compounds a biological activity has already been confirmed (reviewed in Dillier et al. (2006)). Drone and worker larvae of five different age groups (freshly capped and 12, 24, 48, 72 h after cell capping) were extracted for 10 min in 1.5 ml *n*-pentane. These larval stages were chosen because of their different capacity to activate or inhibit the mite oogenesis in the previous experiments (see results in Sections 3.1 and 3.2). For each age group three different extracts with four worker larvae and three drone larvae, respectively, were produced. The extracts were concentrated under a purified flow of nitrogen and added with methyl tridecanoate and hexadecane as internal standards (10 ng/µl each).

The quantification of the injected extracts (2 µl/sample) was performed by GC Varian 3900 (equipped with a splitless injector (250 °C) and Varian Saturn 2100T MS SIM detector) on a capillary column (HP5-MS, length 30 m, internal diameter 0.32 mm, film thickness 25 µm). The temperature of the oven was programmed to increase from an initial setting of 50 °C (3 min) to 280 °C, 10 °C per minute. Temperature was held then at 280 °C for 11 min. Hydrogen was used as carrier gas. Three repetitions were performed for each developmental stage.

2.6. Statistical analysis

We compared the mite reproduction between differentially treated brood cells (Section 2.4) and between differentially treated *Varroa* females (Sections 2.1–2.3) with χ^2 tests on a cross contingency table and an unpaired *t*-test for the number of offspring after confirmation of normal distribution (Kolmogorov–Smirnov-test; WinSTAT Software, R.K. Fitch 2009).

3. Results

3.1. Mite reproduction after natural and artificial infestation of brood cells

The artificial infestation of brood cells with one *Varroa* female did not elicit higher removal rates in the test colonies. Overall, less than 20% of the infested brood cells were removed within the test period of about 8 days. In naturally infested brood cells, about 82% of the invaded *V. destructor* reproduced ($n = 90$; Fig. 2). In drone brood, this fertility rate was similar (79.4%, $n = 68$; Fig. 2). Artificial infestation in recently capped worker brood cells did not affect this percentage significantly (83.5%, $n = 30$; $\chi^2 = 0.019$, $p = 0.57$; Fig. 2). However, the percentages of reproducing *V. destructor* decreased

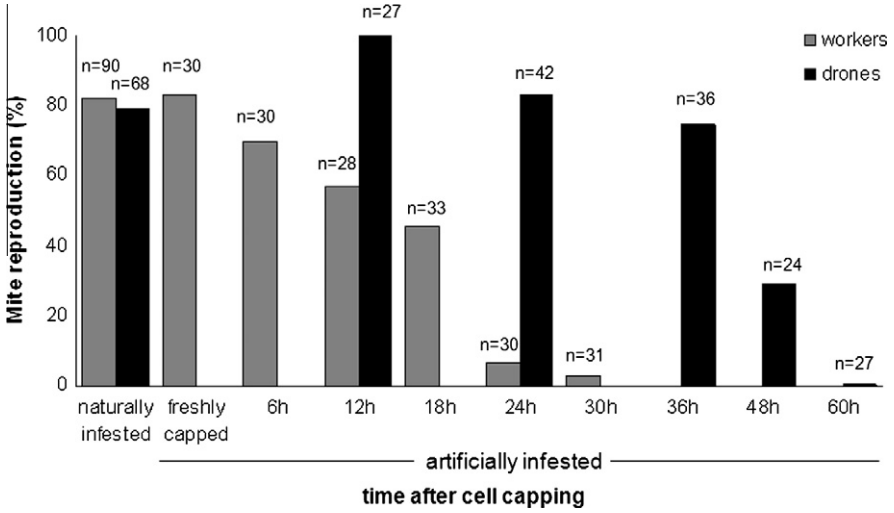

Fig. 2. Percentage of reproducing *V. destructor* females in naturally infested worker and drone brood cells (left two columns) and in brood cells which were artificially infested with phoretic mites at different times after the capping of the brood cells. With increasing time intervals between cell capping and artificial mite infestation the rate of reproducing mites decreased. In worker brood, the rate of reproducing *Varroa* females is significantly reduced already 12 h after cell capping ($n = 28$; $\chi^2 = 5.08$, $p < 0.05$) in drone brood only 48 h after cell capping ($n = 24$; $\chi^2 = 20.05$, $p < 0.01$).

clearly, if the mites were introduced in brood cells 18–60 h after cell capping. In worker brood, mites that were introduced 12 h after cell capping already showed a significantly lower rate of reproduction compared to natural invaded mites ($n = 28$; $\chi^2 = 4.79$, $p < 0.05$). And hardly any of the mites reproduced if they were introduced 24 h and 30 h after cell capping (Fig. 2).

In drone brood, there was only a slight decrease in the rate of reproducing mites if they were introduced in brood cells up to 36 h after cell capping (at 36 h: $n = 36$; $\chi^2 = 0.27$, $p = 0.61$). A significant decrease was only confirmed for those mites that were introduced in drone brood cells 48 h after cell capping ($n = 24$; $\chi^2 = 20.05$, $p < 0.001$). After introduction into brood cells 60 h after cell capping not a single *V. destructor* egg was laid (Fig. 2).

3.2. Reproduction of Varroa mites that have been transferred into brood cells of different ages after initiation of oogenesis

The reproduction of mites that were treated twice (introduced and transferred) was not negatively affected by the handling process: 83.3% of the mites ($n = 30$) that were artificially introduced into freshly capped brood cells and transferred 24 h postcapping to brood cells containing the same larval stage reproduced successfully with an average number of 3.4 ± 1.4 offspring per mother mite (Table 1a). This is equal to the fertility rate to naturally invaded mites (82.2%; Fig. 2).

When mites were transferred from brood cells 24 h after cell capping into cells 48 h after cell capping, the fertility decreased highly significant compared to the control ($n = 36$; $\chi^2 = 14.939$, $p < 0.001$). However, the transfer of *Varroa* mites from brood cells 24 h and 48 h after cell capping, respectively, to brood cells 72 h after cell capping did not reveal a significant effect on the mite's fertility (24–72 h: $n = 29$; $\chi^2 = 2.469$, $p = 0.116$; 48–72 h: $n = 36$; $\chi^2 = 2.377$, $p = 0.123$) albeit the number of mites with unsuccessful reproduction (egg only or male only; Table 1) increased significantly.

Compared to the control, the fecundity (=number of offspring) decreased in all experimental groups, however, only in the case of 24–72 h transfer the differences were significant (*t*-test, $p < 0.01$; Table 1).

Non-reproduction of the introduced mites was not associated with a lack of stored spermatozoa in the spermatheca. In all of the 36 non-reproducing mites from the different approaches we could verify microscopically the presence of sperms.

Table 1
Reproduction of *Varroa* mites that have been introduced into freshly capped brood cells and removed 24 h after cell capping. Mites with activated oogenesis were then transferred to brood cells of different stages. Presented are the reproduction parameters (a) and the results of χ^2-tests for differences in fertility between all groups (b).

Trial	Reproducing mites (fertility) (%)	Avg. progeny per mite (n)	Incomplete reproduction of reproducing mites		
			Egg only (%)	Daughter(s) only (%)	Male only (%)
a					
24 h → 24 h (control, $n = 30$)	83.3	3.4 ± 1.4	8.0	0	4.0
24 h → 48 h ($n = 36$)	36.1	2.5 ± 0.9	7.7	0	0
48 h → 72 h ($n = 36$)	66.7	2.5 ± 1.1	8.3	0	16.7
24 h → 72 h ($n = 29$)	65.5	2.3 ± 1.1	15.8	0	10.5

Trial	24 h → 24 h (control)	24 h → 48 h	48 h → 72 h	24 h → 72 h
b				
24 h → 24 h (control)	–	<0.001**	0.123	0.116
24 h → 48 h	–	–	0.009**	0.018*
48 h → 72 h	–	–	–	0.922
24 h → 72 h	–	–	–	–

* $p < 0.05$.
** $p < 0.01$.

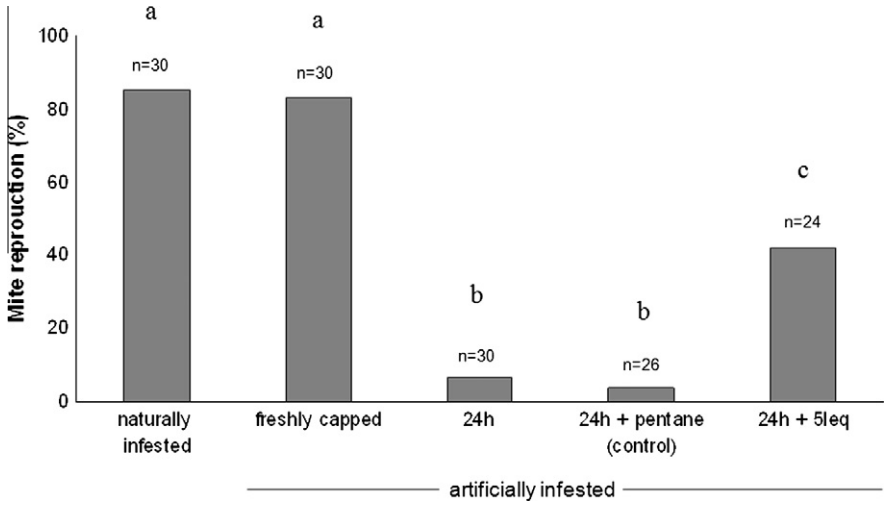

Fig. 3. Effect of the application of a solvent extract from 5th instar worker larvae (five larvae equivalents) on the activation of *V. destructor* reproduction. In naturally invaded brood cells and freshly capped brood cells, artificially infested with a single mite, between 80% and 90% of the mites reproduced. From mites which were introduced 24 h after cell capping, less than 7% reproduced, but after the application of larval extract more than 40% reproduced. Columns followed by different letters differ significantly from each other (a and b: $\chi^2 = 35.6$, $p < 0.001$; b and c: $\chi^2 = 9.2$, $p < 0.01$; a and c: $\chi^2 = 12.01$, $p < 0.01$).

3.3. Effect of the application of larval extract in worker brood cells

In an additional set of experimental setup we could at first confirm the reproduction results presented in Section 3.1 (Fig. 2): The fertility rate of natural invaded and in freshly capped brood cells introduced *V. destructor* females were 85.3% and 83.3%, respectively (Fig. 3). We also confirmed that only about 5% of the mites do reproduce if they were introduced in brood cells 24 h after the cell capping. However, if the larvae in these 24 h brood cells are treated with five larval equivalent of a pentane extract of freshly capped larvae, the percentage of reproducing *V. destructor* increased significantly ($\chi^2 = 9.2$; $p < 0.01$) to more than 40%; the application of the solvent alone had no effect (Fig. 3).

3.4. Chemical analysis of worker and drone larvae extracts

Five different fatty acid methyl esters (FAME: methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate) and three fatty acid ethyl esters (FAEE: ethyl palmitate, ethyl stearate, ethyl oleate) were quantified in the cuticular extracts from *A. mellifera* worker and drone larvae (Fig. 4). The amount of the single compounds varied among age and sex: in freshly sealed drone larvae the total amount of methyl esters were about four times higher compared to worker extracts, the total amount of ethyl esters were about two times higher (Fig. 5). Regarding the larval age, the ratio between FAME and FAEE revealed considerable differences during the first 72 h after cell capping. While at the

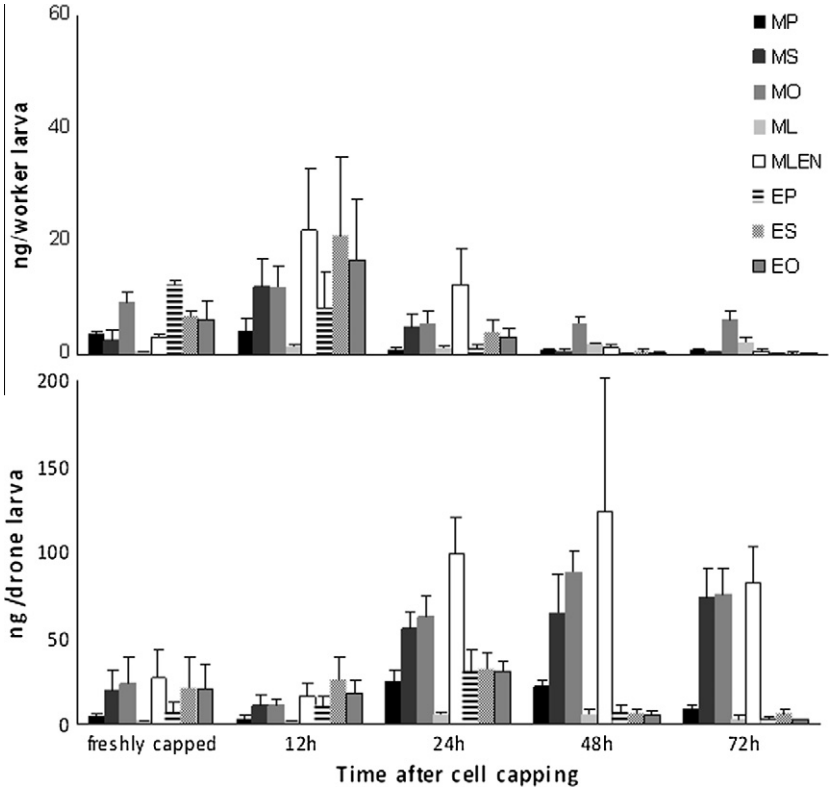

Fig. 4. Comparative amounts of the different fatty acid esters present in the cuticle of freshly capped worker and drone larvae and in larvae 12, 24, 48 and 72 h after cell capping. *MP* methyl palmitate, *MS* methyl stearate, *MO* methyl oleate, *ML* methyl linoleate, *MLEN* methyl linolenate, *EP* ethyl palmitate, *ES* ethyl stearate, *EO* ethyl oleate.

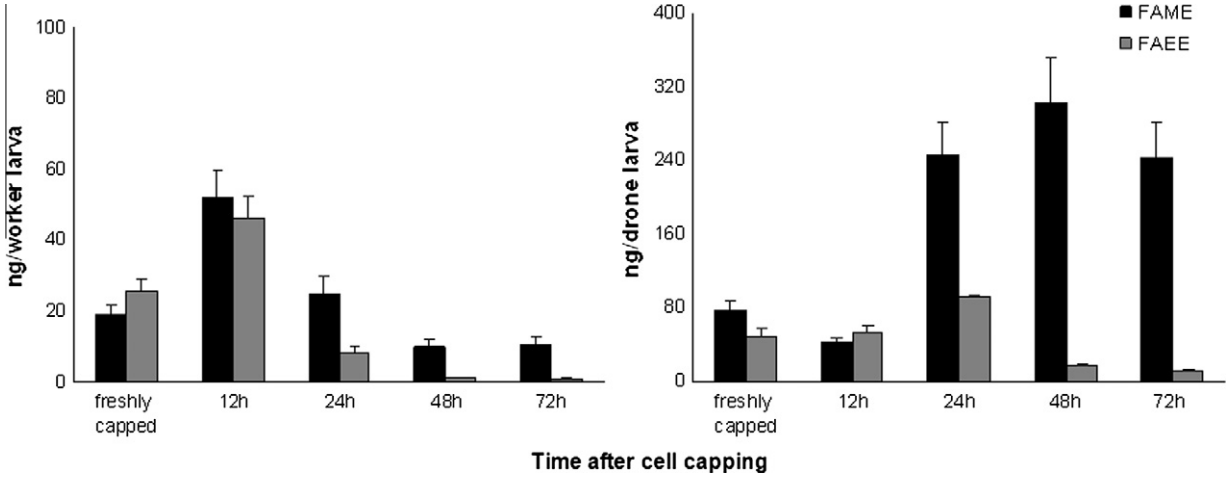

Fig. 5. Averages of total amounts ($n = 3$ for each larval stage) of FAME (Fatty Acid Methyl Ester) and FAEE (Fatty Acid Ethyl Ester) in worker (left) and drone (right) larvae within the first 3 days after capping of the brood cells.

beginning of the capping period FAME and FAEE were present in similar proportions, the amount of FAEE decreased strikingly in worker larvae 24 h and drone larvae 48 h after cell capping. In larvae 72 h after cell capping only traces of FAEE were detectable in both, worker and drone larvae (Figs. 4 and 5).

4. Discussion

The suppression of the reproductive ability of *Varroa* females by the host is still considered a crucial character to affect the mite's population dynamic (Fries et al., 1994; Rosenkranz and Engels, 1994; Correa-Marques et al., 2003; reviewed in Rosenkranz et al. (2010) and Locke and Fries (2011)). In our experiments, we could clearly show that stage specific factors of the host larva are involved in the triggering of the mite's reproduction. At first, the rate of reproducing *Varroa* females decreased significantly if they were introduced into brood cells 18 h (worker) and 48 h (drones), respectively, after cell capping. This demonstrate that only worker larvae within the first 12 h after the cell capping and drone larvae within the first 36 h after cell capping, respectively, possess the entire capacity to activate the reproduction of *V. destructor* females. Hence, we could define for the first time the exact developmental larval stages for both workers and drones that contain the crucial signals to activate the *Varroa* oogenesis.

The nature of these signal(s) still requires final clarification. There is clear evidence that polar compounds of the cuticle of freshly capped larvae are involved in the early step of activation (Garrido and Rosenkranz, 2004). This is confirmed by our application of larval extract to worker brood cells 24 h after cell capping: In untreated or solvent treated brood cells of this stage less than 5% of the introduced *Varroa* mites reproduced whereas more than 40% of the mites start reproduction after application of the larval extract. The significant lower fertility rate in the treated brood cells compared to naturally invaded mites indicates that further factors such as nutritional signals from the larval hemolymph are required to initiate and perform the complete reproductive program.

The results of this study suggest that the female mite synchronizes its reproduction with the ontogenetic development of the host larvae. From an adaptive point of view we can assume a selection pressure on an immediate start of mite oogenesis after invasion of a brood cell. For this purpose, volatiles of the host larvae could be used (Garrido and Rosenkranz, 2004) because they can be perceived even during the first hours after invasion of the brood cell when the mite is stuck to the larval food (Ifantidis et al., 1988). Under natural condition the trapped mites are released from the larval food within 1–6 h after sealing of the brood cell (Ifantidis et al., 1988). Therefore, it cannot be excluded that nutritional factors of the larval hemolymph are additionally involved in this first step of activation.

The further reproductive course of *Varroa* females is likewise influenced by the host larvae. This has already been shown for the sequence of sexes of the mite's offspring (Garrido and Rosenkranz, 2003) and for the number of offspring (Martin and Cook, 1996). With our transfer of mites between brood cells of different stage we could confirm for the first time that an already initiated oogenesis of the *Varroa* female can be interrupted by host factors. If *Varroa* females are transferred from a brood cell 24 h after cell capping – at this stage reproducing mites have already completed oogenesis resulting in a oocyte of about 300 μm (Steiner et al., 1994) – nearly 2/3 of the mites stopped their reproduction and did not lay any egg, while in the control only 17% of the mites remained infertile. Again, this makes sense from an evolutionary point of view: If the development of the oocyte is delayed compared to the development of the larvae there will be hardly a chance to finish the complete development of at least one male and one female offspring within the capping period of the brood cell. Under these conditions it might be adaptive to degrade the growing oocyte by oosorption (Steiner et al., 1995) and, therefore, save resources for the next reproductive cycle. This stop signal seems to be present in the larvae 48 h after cell capping but not to the full extent in larvae 72 h after capping. A transfer of *Varroa* females from brood cells 24–72 h after capping increased the proportion of non-reproducing *Varroa* females only slightly, however, it significantly reduced the fecundity of the transferred mites (number of offspring) and increased the proportion of mites with non-successful reproduction (i.e. egg only, male only, no male). This effect was obviously not associated with a lack of sperm: in a randomly collected proportion of about 25% of the transferred mites all dissected spermathecae were filled with sperms. This is in accordance with our previous observations (Garrido, 2004) and recent results of Kirrane et al. (2011).

Because of the transfer experiment results, we assume that oosorption leading to a temporary infertility of the *Varroa* female is only possible during the phase of oogenesis until the end of the blastoderm stage (0–48 h after cell capping; Steiner et al., 1994). At later stages a degradation of the already formed embryo may not be possible any more. This is confirmed by the results of the transfer of *Varroa* females from brood cells 48 h after capping to 72 h where we did not observe a significant reduction, neither in fertility nor in fecundity.

It is likely that both, semiochemical and nutritional signals of the host are involved in the triggering of the reproductive cycle. So far, we do not know much about the nutritional requirements of reproducing mites except the fact that proteins of the host larvae are stored directly in the ovary of the mite (Tewarson and Engels, 1982).

Considerable more data exist on the effects of stage specific cuticular volatiles of the honey bee larvae. Certain aliphatic esters play an essential role in the brood recognition by the nurse bees (Le Conte et al., 1990, 1994) and are also involved in the host finding of reproductive *Varroa* females (Le Conte et al., 1989, 1994; Trouiller et al., 1992). The secretion of these esters by the honey bee larva reveals a clear ontogenetic pattern with a maximum at the time of cell capping and a significant decrease during the following days (Trouiller et al., 1991, 1992). However, these results focus on the period before and during the cell capping and the associated invasion behavior of female *V. destructor*. A quantitative analysis of these cuticular compounds in relation to the mite's reproduction has not been published so far. We here analyzed in detail the quantitative pattern of 5 methyl and 3 ethyl esters on the larval cuticle from those larval stages that are supposed to have either an activating or inhibiting effect on the mite's reproduction. Our quantification revealed a similar pattern of FAME and FAEE within the freshly sealed larvae but lower total amounts per larvae compared to Trouiller et al. (1992) which might be due to different extraction methods. Surprisingly, the decrease of total esters within the first 2–3 days after cell capping is mainly caused by the FAEE while the amount of FAME is reduced only slightly or, in the case of drone brood, the amount is even increasing. In both worker and drone larvae, there is a striking decrease of FAEE exactly in those larval stages that are no longer able to activate the reproduction of *V. destructor*, i.e. worker larvae 24 h and drone larvae 48 h after cell capping. FAEE could therefore be involved in the first activation process for *Varroa* reproduction. This is rather speculative at the moment and requires the confirmation of a causative correlation; however, it has already been shown that certain FAME and FAEE are used by *V. destructor* as kairomone (Le Conte et al., 1989, 1994; Trouiller et al., 1992) and moreover are part of the *Varroa* female's sex pheromone (Ziegelmann et al., under review). Even though these aliphatic esters represent promising candidates it should be noted that other compounds like hydrocarbons from

the non-polar fraction of the larval cuticle could be additionally involved in the chemotactic control of mite reproduction.

So far, we do not have a reliable suggestion for the nature of the stop signal. Heptadecene is the only semiochemical which is considered to have a fecundity-reducing potential in *V. destructor* (Nazzi et al., 2002) and is obviously produced by the honey bee larvae under stress conditions. The results of our control experiments do not indicate a stress reaction of the larvae after the artificial introduction of mites so that it is unlikely that this compound is involved in the here described interruption of mite's reproduction.

Except for the suggestion of the FAEE as a potential initial activator of the mite's oogenesis, we cannot specify the signals involved in the host derived triggering of *Varroa* reproduction. For further research, an *in vitro* system allowing artificial feeding of reproducing mites is urgently needed. Since the work of Bruce et al. (1988, 1991) on artificial feeding of mites, no promising approach has been undertaken.

Our experiments confirm once again that the reproduction of the parasitic mite *V. destructor* is activated by host factors of the honey bee larva. This has already been assumed by Trouiller and Milani (1999) who stimulated the reproduction of *Varroa* within artificial gelatin cells by application of an extract of 5th instar larvae. Later on, Garrido and Rosenkranz (2004) showed that volatiles emitted by the freshly capped larvae are involved in the activation of the mite's oogenesis.

We here used a within colony approach to verify these results under natural conditions. The introduction of mature *Varroa* females into honey bee brood cells has been used successfully for many years at our lab. The introduction of phoretic mites – collected from nurse bees – into freshly sealed brood cells obviously resembles the situation of natural invaded mites. We did not record any differences in the fertility of artificial introduced and natural invaded mites. This confirms former results using this well-established technique (Rosenkranz and Stürmer, 1992; Rosenkranz and Bartalszky, 1996; Martin and Cook, 1996; Garrido and Rosenkranz, 2003) however it contradicts recent results from Kirrane et al. (2011) where nearly all of the introduced phoretic mites (and most of the transferred brood mites) remained infertile. So far, we do not have a satisfying explanation for these differences.

Our results provide an important tool for the further analysis of the crucial host signals by analyzing those host stages that have the capacity to activate or inhibit the *Varroa* reproduction. This may also help to better understand the recently confirmed genetical basis of non-reproduction in *V. destructor* (Behrens et al., 2011).

Acknowledgments

Funded by the European Commission BEE DOC Project (EU Contract Number: FP7-KBBE-2009-3 244956 CP-FP) and by the German Ministry for Education and Science (BMBF) Project FUGAPIS (Project Number: 0315125A).

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