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Original article

The mating behavior of *Varroa destructor* is triggered by a female sex pheromone. Part 2: Identification and dose-dependent effects of components of the *Varroa* sex pheromone

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Abstract – Reproduction of female *Varroa destructor* happens within the sealed brood cell of the honeybee host. The mating represents the last step of the reproductive cycle and is usually performed between the mature male offspring and one or more daughter mites. By offering solvent extracts of freshly molted females to male *Varroa* mites in our mating bioassay, we could clearly confirm the presence of a volatile female sex pheromone. After separation of the extract into a polar and non-polar fraction, only the polar fraction elicited the typical mating behavior of male mites. GC-MS analysis of the active fraction revealed a pattern of three fatty acids as the main components and the respective ethyl esters. We could prove that all these substances stimulated the male mating behavior, and we present results on the dose-dependent reactions of the males toward these compounds. The identification of a *Varroa* sex pheromone might enable new options for a biological control of the parasite.

***Varroa destructor* / mating behavior / sex pheromone**

1. INTRODUCTION

The life cycle of *Varroa destructor* is closely linked to its honeybee host, without any free-living stage (Rosenkranz et al. 2010). This is eminently true for the reproduction of the female mites that can only happen within the sealed honeybee brood cells. For the reproductive success of *Varroa*, it is therefore essential to (1) find a suitable host larva and (2) to complete oogenesis, preimaginal development, and mating of the offspring within the limited time of honeybee brood development. For the close synchronization of the *Varroa* reproduction with the brood development of the host, chemical cues play an indispensable role. This has already been already confirmed for the host finding behavior that represents the first challenge for mother mites that are ready to start the reproductive cycle. These already inseminated females preferentially parasitize nurse bees (Kraus 1993; Kuenen and Calderone 1997) in order to be transported to unsealed brood cells. It is assumed that these nurse bees are selected by means of an age-dependent hydrocarbon pattern of the bee's cuticle (Chiroudi et al. 1997); Del Piccolo et al. (2010) identified (Z)-8-heptadecene as a crucial compound in the selection of the suitable bee stage. The invasion of the suitable brood cell is triggered by the

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kairomonal effect of several fatty acid methyl and ethyl esters of the fifth instar bee larvae (Le Conte et al. 1989, 1994; Troullier et al. 1991, 1992). After entering the brood cell, the start of the mite's oogenesis is immediately activated by yet unknown volatile compounds of the host larva (Milani and Chiesa 1990; Troullier and Milani 1999; Garrido and Rosenkranz, 2004).

It is likely that the mating behavior of *V. destructor* is also triggered by chemical cues. The mating between the mature male offspring and one or more daughter mites represents the last step of the *Varroa* reproductive cycle and has to be completed within the brood cell before the hatching of the bee. Non-mated daughter mites either seem to die and disappear from the phoretic mite population (Garrido 2004) or should at least be incapable of laying fertilized eggs during the next reproductive cycle. Therefore, daughter mites have to mate immediately after the adult molt when they become sexually mature. That sort of behavior has been described previously (Donzé and Guerin 1994; Donzé et al. 1996) and has been further specified by Ziegelmann et al. (2012) in a laboratory mating bioassay. Ziegelmann et al. (2012) could indeed demonstrate that female mites become attractive to males exactly during the adult molt. In contrast, the female deutochrysalis stage which precedes the adult stage is not attractive to male mites, while the attractiveness of older adult females decreases significantly with age.

So far, the cues triggering the mating behavior within the dark and tight brood cell are unknown. However, we suppose that pheromones released by the young female mite are involved to distinguish females of different ages and to elicit the male's mating behavior. The identification of such a *Varroa* sex pheromone could possibly be used to disrupt the mating behavior and therefore would be of great importance for the practical beekeeping. The present paper reports on bioassays and chemical analyses of solvent extracts from *Varroa* females, which have been shown to be most attractive to males in the previous experiments (Ziegelmann et al. 2012).

2. MATERIALS AND METHODS

2.1. Collection of mites

Male and female *Varroa* mites were obtained from brood combs of heavily infested *Apis mellifera* colonies at the Apicultural State Institute, University of Hohenheim in Germany. Sexually mature males and freshly molted adult females as well as female deutochrysalis can be found in brood cells 8 to 9 days after capping. Therefore, brood combs containing pupae of suitable age were taken to the laboratory, and the brood cells were opened with forceps. The required mite stages (female deutochrysalis, freshly molted adult females, adult males; for photographs of these stages see Rosenkranz et al. 2010) were transferred into queen cell cups (Nicot system®, Karl Jenter, Metzingen) and kept at 28–30 °C for maximum 2 h in order to prevent a decrease in vitality and mobility.

2.2. Extraction of female *V. destructor*

Extracts for behavioral assays were prepared in screw-top vials by immersing about 20 young freshly molted female mites, which have been found to be attractive in the bioassay, in diethyl ether (Roth, GC grade ≥99.8 %, stabilized with ~1 % ethanol) for 2 weeks at −20 °C. Five microliters of solvent per individual were used. After 2 weeks, the extracts were transferred into new vials and stored at −20 °C until use. Diethyl ether was used because other solvents appeared to be unsuitable in preliminary tests (Lindenmayer 2007). For quantitative analyses, we prepared diethyl ether extracts with extraction times of 7 days, 1 h, and 1 min in the same manner.

2.3. Fractionation of extracts with column chromatography

Ether extracts of young attractive females were fractionated on a silica gel micro column. Dry silica gel (0.25 g; Silica gel 60, 0.06–0.2 mm, Roth) was suspended with pentane in a test tube. After pouring the slurry into the column (7 cm length, 0.8 cm I.D., 3.5 mL), the silica gel was washed with 10 mL pentane. The female extracts were evaporated to 5 µL and redissolved with 195 µl pentane. The extract was then pipetted on top of the column and eluted

sequentially with 2 mL pentane and 2 mL diethyl ether. The obtained pentane and diethyl ether fractions were evaporated to a concentration of one female mite equivalent per 5 μL and stored at −20 °C until use.

2.4. Mating bioassays

The responses of male *Varroa* mites to extracts, fractions, and pure substances were measured by using the "mating bioassay" described in detail in Ziegelmann et al. (2012). For the application of the extracts, the test was slightly modified: total extracts, fractions, and pure substances were applied to a piece of filter paper (size: 1.5×15 mm) with a 10-μL Hamilton syringe. The filter paper was folded in the middle and placed at the edge of the plastic cell cups (Nicot System®, Karl Jenter, Metzingen, 9 mm inner diameter) that served as a test arena. A living female deutochrysalis was placed at the bottom of the cell cup at 3.5 mm distance to the filter paper. This immobile ontogenetic stage has been proved to be completely non-attractive to the male mite and was therefore used as a dummy. After three tests, the dummies and the cell cups were replaced by new ones. The male responses towards the deutochrysalis were categorized as follows: (1) movement towards/around the deutochrysalis and palpating it, (2) mounting the dorsum of the deutochrysalis, and (3) copulation attempts on the venter of the deutochrysalis. The duration of each of these behaviors was recorded over a period of 5 min using the Observer 2.0 software (Noldus Information Technology). All tests were performed at temperatures of 28–30 °C at the bottom of the test arena. This temperature was chosen because the preference temperature of *Varroa* mites is clearly lower compared to the temperature of the honeybee brood (Le Conte and Arnold 1988; Rosenkranz 1988). Our experience from former laboratory bioassays confirmed an optimal activity of *Varroa* mites under these temperature conditions.

2.5. Bioassays with total extracts of young females, fractions of the extracts, and pure substances

The biological activity of extracts of young females was tested by exposing males to defined amounts of total extract and one living deutochrysalis. The extract was applied to the filter paper in doses of two female equivalents; in control tests, the filter paper was treated with diethyl ether only. Pentane and ether fractions were tested in concentrations of five female equivalents in the same way. The higher concentration was applied in order to equalize possible losses of active substances during the column fractionation. Single substances that were identified from the active fraction (see Section 2.6) were purchased (Sigma Aldrich, GC grade), dissolved in diethyl ether, and were tested in doses of 1, 10, 100, and 1,000 ng.

2.6. Chemical analysis

Structural analysis of the volatile compounds was performed on a Shimadzu GC-MS combination (GC-17A/GCMS-QP 5050A) equipped with a split/splitless injector and a fused silica DB-5 column (30 m× 0.25 mm I.D., 0.25 μm film thickness). The analyses were operated in the splitless injection mode with the following conditions: 60 °C isothermal for 2 min, followed by a temperature increase at a rate of 4 °C/min up to 300 °C that was held for 12 min. The carrier gas was helium at 1.6 mL/min, and temperatures of the injector and detector were 240 and 280 °C, respectively. For each analysis, 1.5 μL of the extract corresponding to five *Varroa* female equivalents was injected. The compounds of the active fraction were identified by analysis of the mass spectra and retention time followed by co-injection of the identified authentic standards (Sigma).

Quantitative analyses were performed on a Varian GC-MS combination (GC: Varian 3900; detector: Varian Saturn 2100 T MS SIM) equipped with a splitless injector and a fused silica DB-5 column (30 m×0.25 mm I.D. and 0.25 μm film thickness). The oven temperature was programmed from 50 °C (isothermal for 3 min) to 280 °C (held for 11 min) at a rate of 10 °C/min. The carrier gas was helium, and the temperatures of the injector and detector were 250 and 280 °C, respectively. For the quantitative analysis of the fatty acids ethyl esters, 1.5 μL of the original extract corresponding to five *Varroa* female equivalents was injected and the peak areas compared with the respective authentic standard analyzed under the same experimental conditions. Free fatty acids in the extracts were derivatized with *N*,*O*-

Bis(trimethylsilyl)trifluoroacetamide/BSTFA (Supelco 33148) into trimethylsilyl esters prior to GC analysis.

2.7. Data analysis

Behavioral data were analyzed with the SPSS 15.0 statistics software using one-way ANOVA followed by Bonferroni correction. Data sets were checked for normal distribution by Kolmogorov–Smirnov test. In cases of non-normal distribution, a log transformation was performed. If normal distribution could not be achieved, the nonparametric Kruskal–Wallis test was used. The respective tests are given in the results. Differences between groups with $P<0.05$ were considered statistically significant.

3. RESULTS

3.1. Male responses to total extracts of young females and fractions

Male *V. destructor* did not show any copulatory responses when they were exposed to deutochrysalis and filter paper treated with solvent. Only few single males mounted the female dorsum or moved to the female's venter for a few seconds, but did not show any clear copulation attempts (Figure 1). In contrast, when males were exposed to filter paper containing the total extract of young females, mounting behavior and copulation attempts with the deutochrysalis took place, resembling the typical mating behavior towards young females (Ziegelmann et al. 2012). However, in the venter-to-venter position, males did not pause at the gonopore area on one side but moved from one gonopore to the other. Male responses were highly significant for all three behavioral categories (Kruskal–Wallis, $P<0.001$, $n=30$). Males spent one fifth of the test time with copulation attempts.

In bioassays with fractions of ether extracts of young adult females, the diethyl ether fraction induced significantly more and longer responses of the male for all three behavioral categories than the pentane fraction (Figure 2; walking around female, ANOVA, $P<0.005$, $n=24$; mounting, ANOVA, $P<0.05$, $n=24$; copulation attempts, ANOVA, $P<0.05$, $n=24$). Thereby, the mean duration of copulation attempts of the ether fraction was comparable to responses observed in the presence of the total extract. In contrast, no clear copulation attempts occurred in the presence of the pentane fraction.

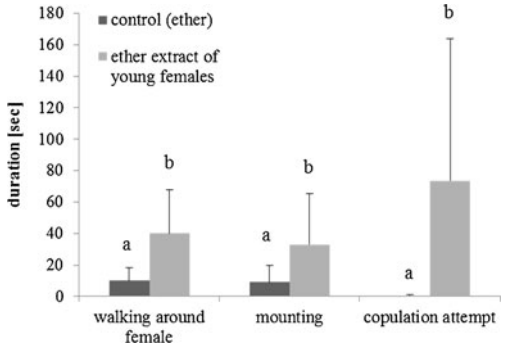

Figure 1. Response of males of *V. destructor* to a female deutochrysalis in the presence of diethyl ether extracts of freshly moulted females applied on filter paper. Pure diethyl ether was used in the control experiments. Extracts were applied in dosages corresponding to two female equivalents. *Means with different letters* are significantly different (Kruskal–Wallis; $P<0.001$; $n=30$).

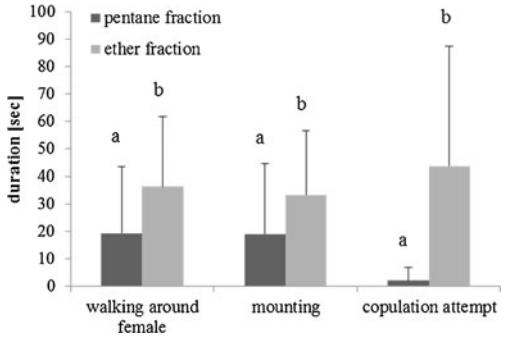

Figure 2. Response of males of *V. destructor* to a female deutochrysalis in the presence of fractions of diethyl ether extracts of freshly moulted females. One female deutochrysalis was offered with filter paper either treated with the pentane fraction ($n=24$) or the ether fraction ($n=24$) of the extracts. The used dosages correspond to five female equivalents. *Means followed by different letters* are significantly different (ANOVA; $P<0.05$; $n=24$).

3.2. Chemical analysis

Qualitative GC-MS analysis of the behaviorally inactive pentane fraction revealed a pattern mainly composed of uneven *n*-alkanes with chain lengths of 21–29 carbon atoms and the respective alkenes and methyl alkanes. The biological active polar fraction was dominated by the fatty acids oleic acid, palmitic acid, and stearic acid, as well as their respective ethyl esters (Figure 3). Quantitative analyses revealed that the proportion of the fatty acids and fatty acid ethyl esters differ with the extraction time (Table I). In long-term extracts (1 week), oleic acid was clearly the main component with 61 ng/female and about 50 % of the active substances within the polar fraction. Palmitic acid and stearic acid accounted for 32 ng/female (26 %) and 25 ng/ female (21 %), respectively, whereas the fatty acid ethyl esters were found in smaller quantities of about 2 ng/female. In the 1-h extract, oleic acid was found in smaller amounts (17 ng/ female corresponding to 33 %), while the relative amount of palmitic acid (36 %) and stearic acid (28 %) increased. In the short-term extract of 1 min, still 5 ng of oleic acid could be found per female mite (20 %), while palmitic and stearic acid were found in higher amounts of 12 ng/female (46 %) and 8 ng/ female (33 %), respectively; the ethyl esters were only present in traces.

3.3. Male responses to pure substances

In bioassays with synthetic substances identified in the ether fraction, all fatty acids and fatty acid esters were biologically active and elicited the male mating behavior. Copulatory responses to oleic acid were significantly highest at a dosage of 10 ng and lowest at 1,000 ng (Figure 4a, Table II). For ethyl oleate, the same trend was evident (Figure 4b), albeit without significant differences. Palmitic acid, ethyl palmitate, and ethyl stearate also showed maximum copulatory responses at 10 ng and lower responses to other concentrations, but the differences were also not significant (Figure 4c, d, f). In bioassays with stearic acid, the differences between the copulatory responses to the four dosages were low, and the maximal copulation attempts at a dose of 1 ng were lower compared to the other compounds (Figure 4e).

4. DISCUSSION

In our previous experiments with freshly moulted young *Varroa* females, we demonstrated the exceptional attractiveness of this female

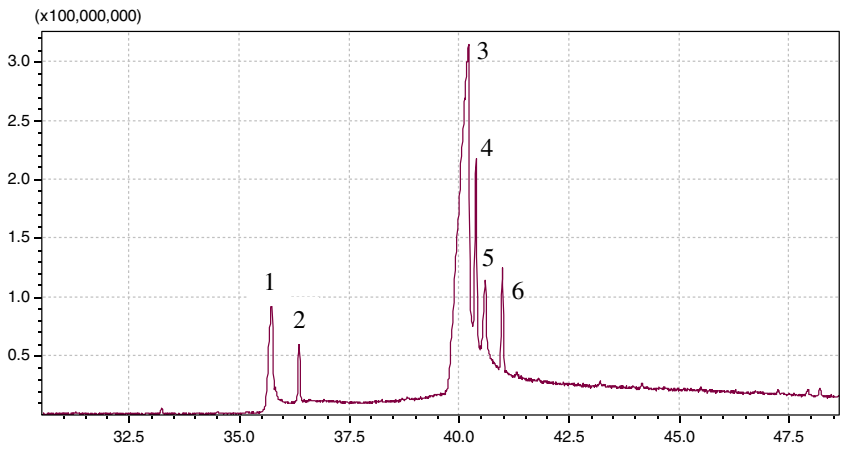

Figure 3. Total ion chromatogram of the diethyl ether fraction of a diethyl ether extract of young females of *V. destructor*. The single substances were identified based on their mass spectra and by co-injection with pure substances. *1* palmitic acid, *2* ethyl palmitate, *3* oleic acid, *4* ethyl oleate, *5* stearic acid, *6* ethyl stearate.

Table I. Amounts of the biological active free fatty acids and fatty acid ethyl esters in diethyl ether extracts of female *V. destructor* shortly after the adult moult.

Extraction time	Concentration (ng/female) (%)					
	Palmitic acid	Stearic acid	Oleic acid	Ethyl palmitate	Ethyl stearate	Ethyl oleate
7 days	32.3 (26.3)	25.4 (20.9)	60.7 (50.0)	0.5 (0.4)	0.6 (0.5)	2.2 (1.8)
1 h	18.9 (35.5)	14.8 (27.9)	17.3 (32.6)	0.8 (1.5)	0.6 (1.1)	0.7 (1.3)
1 min	11.6 (46.3)	8.1 (32.6)	5.0 (20.1)	< 0.2	< 0.2	< 0.2

Extraction times varied between 1 min and 7 days. Values below 0.2 ng/ female were not quantified in detail

stage to male mites (Ziegelmann et al. 2012). In the present study, males showed the typical mating cascade and made significant copulation attempts when exposed to diethyl ether extracts of young females together with a deutochrysalis serving as an unattractive dummy. The applica-

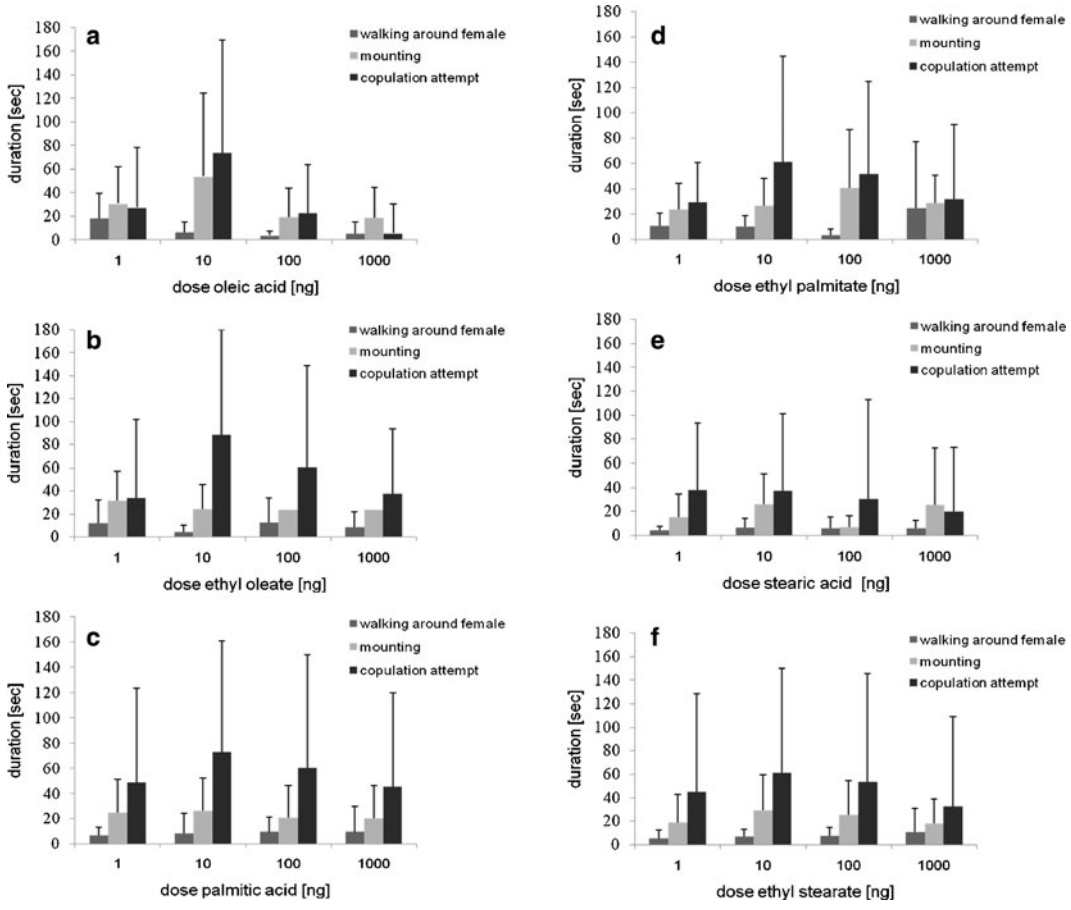

Figure 4. Response of males of *V. destructor* to a female deutochrysalis in the presence of single substances identified in the diethyl ether fraction of extracts of young females. Substances were dissolved in diethyl ether and applied in absolute amounts of 1, 10, 100, and 1,000 ng on filter paper.

Table II. Statistical comparisons of the responses of males of *V. destructor* to a female deutochrysalis in the presence of oleic acid, the main component of the active fraction used in the bioassay (see Figure 3a).

Dose (ng)	*n*	A. Copulation attempt				
		Mean (s)±SD	Significance			
			1 ng	10 ng	100 ng	1,000 ng
1	24	27.2±51.0	–	0.182	0.785	0.002**
10	24	73.8±95.4	–	–	0.104	<0.001***
100	24	22.6±40.7	–	–	–	0.004**
1,000	24	5.1±24.2	–	–	–	–

Presented are the mean durations of mounting (a) and copulation attempts (b) for applications of 1, 10, 100, and 1,000 ng on filter paper and the corresponding *P* values

**$P<0.01$, ***$P<0.001$, differences between groups were considered statistically significant (Kruskal–Wallis)

tion of the solvent alone did not elicit any specific behavior of the tested males toward the deutochrysalis. Because the male mites made definite copulation attempts with the unattractive and immobile deutochrysalis when extracts of young female mites were offered on filter paper in a distance of several millimeters, the active substances must be volatile. Under the conditions within the tight brood cell, this should enable male mites to perceive attractive young female mites. This confirms that volatile compounds of young adult female mites are the crucial cue stimulating mating behavior of the males, demonstrating for the first time the existence of a female sex pheromone in *V. destructor*.

By use of a simple column chromatography, we separated the extract into one fraction containing six polar compounds and a non-polar fraction that mainly contained typical cuticular hydrocarbons (Nation et al. 1992). Only the polar fraction elicited the typical male mating behavior ranging from searching, mounting, to copulation attempts indicating that the biological active compounds are only present in this fraction.

The qualitative GC-MS analysis of the polar fraction revealed three fatty acids as main components and their corresponding ethyl esters in clearly lower amounts. In our bioassays, all these substances stimulated the male mating behavior which demonstrates that they are part of the *Varroa* sex pheromone. These substances are ubiquitous in arthropods and are all derived from the lipid metabolism (Stanley-Samuelson et al. 1988). Their function as semiochemicals is well known, and also, their role as sex pheromones was confirmed for several arthropods. Free fatty acids have been described as part of sex pheromones in some ixodid ticks (Sonenshine 2004), and the here identified fatty acids can be found in the anterior reproductive tract of female ticks of the genus *Amblyomma* (Allan et al. 1991) and *Dermacentor* (Allan et al. 1988).

Fatty acids and the respective methyl and ethyl esters have been proved to play a crucial role in the intraspecific communication in honeybees, but are also of utmost importance for the host–parasite relationship between *Varroa* females and the honeybee larvae. A releaser effect as brood pheromone has been described for a blend of ten fatty acid esters, including ethyl palmitate, ethyl stearate, and ethyl oleate (Le Conte et al. 1990, 2001). The same compounds of the larval cuticle are used by reproductive *Varroa* females for the host finding (Le Conte et al. 1989; Rickli et al. 1992). Additionally, short-chain fatty acids from the larval food can attract (Nazzi et al. 2004) or repel (Nazzi et al. 2009) *Varroa* females. After the invasion into a brood cell, also the start of

the reproduction of the *Varroa* female is triggered by components of the polar fraction of the larval scent (Troullier and Milani 1999; Garrido and Rosenkranz 2004).

This could indicate that part of the active substances that have been analyzed from extracts of our female *Varroa* mites derive from the host pupa. However, there are several facts that contradict such an assumption. First, Troullier et al. (1991, 1992) showed that the methyl and ethyl esters on the larval cuticle reach maximal values at the time of cell capping, and the amount of these esters decreases rapidly after capping. In white-eyed drone pupae, for instance, fatty acid esters are only present in traces. We confirmed this by additional quantitative analysis of extracts of worker pupae 8 days after cell capping. At this developmental stage, only small amounts of fatty acid methyl esters but hardly any ethyl esters are present (Frey et al., personal communication). Second, in an additional control experiment we could demonstrate that the presence of the pupa alone does not elicit the mating behavior (Ziegelmann, unpublished data). Finally, Martin et al. (2002) already confirmed the existence of palmitic acid, stearic acid, and oleic acid in the headspace of adult *Varroa* females but could not detect oleic acid in methylene chloride extracts of pupae 5 days after capping.

Thus, we can exclude that the identified compounds derive from the bee pupae. The clearly higher amounts of the sex pheromone components in the long-term extract (7 days) compared to the short-term extracts (1 h, 1 min) indicate that the substances are present within the mite's body and are gradually released to the surface of the cuticle. It can therefore be assumed that the short-term extracts represent a more realistic proportion of the compounds in the headspace compared to longer extraction times. Moreover, a gradual release of the active compounds is also supported by previous experiments showing that the release of the female sex pheromone starts during molt, in the very moment when the exuvia is removed and the gonopores are uncovered. Afterwards, the activity of the pheromone decreases within 24 h (Ziegelmann et al. 2012). The reason for the extreme decrease might be induced through a stop of pheromone production, through an inhibition of the release through the gonopores, or by the synthesis of an anti-aphrodisiac substance by older females after mating.

The quantification of the substances showed that in all extracts the fatty acids are dominant compared to the ethyl esters. However, at this time we do not know the exact quantity and composition of the pheromone blend emitted by the *Varroa* females of different ages. The differences in the amounts and the proportions of the compounds according to the extraction time demonstrate that headspace analysis by use of closed-loop stripping apparatus or solid-phase microextraction (Schulz et al. 2004) is required to identify the airborne blend of the *Varroa* sex pheromone.

In our bioassays, all six identified fatty acids and ethyl esters of the polar fraction do elicit the cascade of the male's copulation behavior. The maximum responses toward all compounds were achieved with an application of about 10 ng (Figure 4). At least for the fatty acids, this is within the range of the amount quantified for a single female mite (Table I). The fact that all compounds elicited the mating behavior might indicate a redundancy of the pheromone signal. Under the condition within a closed brood cell and usually brother–sister mating a "fine-tuning" of the pheromonal communication as in different moth species might not be necessary (Wyatt 2003). This, however, must be proved by the application of mixtures with variable numbers and quantities of the six compounds. The exact quantification of the airborne blend within the brood cell and the development of a suitable bioassay to prove possible synergetic, additive, or redundant effects of the single compounds will be a main challenge for further approaches.

For oleic acid, we observed the highest copulatory responses with a maximum duration of copulation attempts at 10 ng. However, our results with the pure substances reveal that even high dosages of the pheromone do not deter males from copulation attempts and still have an attracting effect on males.

The identification of the active compounds of the *Varroa* sex pheromone enables new options for the control of these mites based on semiochemicals. These could consist in the introduction of the compounds in supernatural concentrations into brood cells to stimulate a repellent effect as in other pheromones (Wyatt 1997). However, our present results do rather support an application of the active pheromone blend to an entire *Varroa* "family" leading males to believe there are suitable females everywhere. This might confuse males and disrupt an effective mating behavior, in analogy to the mating disruption strategy to control lepidopteran pests (Witzgall et al. 2008). However, it will be a challenge to apply the substances in a suitable way and to prevent side effects in the host. The here published results will hopefully encourage research activities in this direction.

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Le comportement d'accouplement de *Varroa destructor* est déclenché par une phéromone sexuelle de la femelle. Partie 2: Identification des composants de la phéromone sexuelle de *Varroa* et leurs effets en fonction de la dose

Acari / *Varroa destructor* / comportement d'accouplement / pheromone sexuelle / Apidae

Das Paarungsverhalten von *Varroa destructor* wird durch ein weibliches Sexualpheromon gesteuert. Teil 2: Identifizierung und dosisabhängige Effekte von Komponenten des *Varroa* Sexualpheromons

***Varroa destructor* / Paarungsverhalten / Sexualpheromon**

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