

The mating behavior of *Varroa destructor* is triggered by a female sex pheromone

Part 1: Preference behavior of male mites in a laboratory bioassay

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Received 14 October 2011 – Revised 19 October 2012 – Accepted 7 November 2012

Abstract – For the reproductive success of the honeybee mite *Varroa destructor*, an effective coordination of host finding, oogenesis, and mating is crucial. In order to analyze the mites' mating behavior and the involved cues, a new bioassay was designed and the male behavior towards different female stages compared. This bioassay represents a simple tool for behavioral observations of *V. destructor* in the laboratory, showing that males almost exclusively mated with freshly molted females and that older females were rather unattractive for them. Furthermore, we could show that the highest attractiveness of female mites is limited to a short time period of about 24 h immediately after the adult molt. Our results confirm a selective pressure on effective timing and sequence of the mating behavior, and might provide possibilities for biological *Varroa* control.

Varroa destructor / mating behavior / honeybee / bioassay

1. INTRODUCTION

The hemophagous mite *Varroa destructor* is currently the most serious pest of the western honeybee *Apis mellifera* (Rosenkranz et al. 2010). Colonies without periodic treatment usually collapse within 1–3 years due to the weakening of individual worker bees caused by loss of hemolymph (De Jong et al. 1982; Schneider and Drescher 1987; Schatton-Gadelmayer and Engels 1988) and damage due to immunosuppression

and secondary infections (Yang and Cox-Foster 2007; de Miranda and Genersch 2010; Genersch 2010; Richards et al. 2011). Beekeepers make use of a wide range of chemical and biotechnical methods to keep the mite population below the damage threshold. Despite these intensive treatments, mite infestations cause severe colony losses worldwide every year, and have recently been proven to be the main factor for periodically high colony winter losses (Genersch et al. 2010).

Therefore, further measures are urgently needed to limit the population growth of *V. destructor*. So far, a biological approach to reduce the reproductive success of *Varroa* females is lacking. The reproduction of *V. destructor* is closely synchronized with the life cycle of its host. For their reproduction, female mites have to enter worker and drone brood cells shortly before the capping of the cell, and

Electronic supplementary material The online version of this article (doi:10.1007/s13592-012-0182-5) contains supplementary material, which is available to authorized users.

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Manuscript editor: Yves Le Conte

hide in the larval jelly (Ifantidis 1988; Boot et al. 1992). Oviposition starts 60 to 70 h later with a haploid male egg followed by four to five diploid female eggs at 30-h intervals (Ifantidis 1983, 1990; Ifantidis and Rosenkranz 1988; Rehm and Ritter 1989; Steiner et al. 1994). The period from egg to the adult stage lasts 5.8 and 6.6 days for female and male mites, respectively (Ifantidis 1990; Rehm and Ritter 1989; Martin 1994; Donzé and Guerin 1994). The preimaginal development is subdivided into proto- and deutonymph stages. Before each molt, the mobile nymph stages become immobile; these pharate stages are called protochrysalis and deutochrysalis (Laurent and Santas 1987; Donzé and Guerin 1994).

Both sexes of *V. destructor* become sexually mature with the last molt. The mating takes place within the sealed brood cells. In single infested cells, the daughter mite is inseminated by its brother. Only adult daughter mites leave the brood cell together with the hatching young bees, and only mated females can be found later on within the phoretic mite population (Garrido and Rosenkranz 2003). Therefore, a rapid and effective mating is crucial for the reproductive success of the *Varroa* female. This finds its expression in specific copulation behavior (Donzé and Guerin 1994). Male mites reach maturity first and join the fecal accumulation site, waiting for young adult females. The mating procedure starts as soon as a female arrives. The mating process is completed by the transfer of the male's spermatophore into the female gonopore. Insemination occurs by means of the spermatodactyl, a cannula-like structure that is part of the chelicerae (Evans 1992). Observations by Donzé et al. (1996) and Fahle and Rosenkranz (2005) indicate that male mites preferably mate with young female daughter mites at the fecal accumulation site. These young females are remated until the next daughter female arrives at the fecal accumulation site. With the number of matings, the number of spermatozoa in the spermatheca increases up to 35 (Donzé and Guerin 1994; Donzé et al. 1996).

So far, there is no suitable laboratory bioassay for the quantification of specific behavioral traits of male mites. For this reason, our first objective was to establish a behavioral bioassay to study the mating behavior of male mites. This bioassay was used to identify the most attractive female stages as a basis for further studies on the cues involved in the mating behavior.

2. MATERIALS AND METHODS

2.1. Collection of mites

Male and female *V. destructor* were obtained from heavily infested *A. mellifera* colonies at the Apicultural State Institute, University of Hohenheim in Germany. Sexually mature male mites and daughter females as well as younger female mites can be found in brood cells 8 to 9 days after capping. At this stage, the pupa has brown eyes and a brownish head and thorax. Combs with these pupal stages were taken to the laboratory where brood cells were opened with forceps, and the suitable mite stages were transferred into queen cell cups (Nicot system[®], Karl Jenter, Metzingen). The mites were kept at 28–30 °C for a maximum of 2 h in order to prevent a decline in vitality and mobility. One adult male and different female stages were used for the bioassays. The female stages were selected by means of morphological features: The deutochrysalis is immobile and has a very soft cuticle, and sclerotization is not yet visible. At this early stage of development the deutochrysalis appears mostly white due to the hemolymph shining through the transparent cuticle. The legs are stretched out forward, and the deutochrysalis lies planar on the ground. The late-stage deutochrysalis (shortly before the molt) already shows sclerotized areas on the new cuticle in the form of a thin rose ring along the edge of the opisthosoma. Only the first pair of the deutochrysalis' legs is still stretched out forward, the other legs lie beneath the body, whereas the anterior part of the body adopts a slightly upward position. Freshly molted females are mobile and light brown in color, whereas old females are dark brown mother mites (for photographs of adult male, early-stage deutochrysalis, freshly molted female, and old female, see Rosenkranz et al. 2010; late deutochrysalis see Figure 4a).

2.2. Mating bioassay

Bioassays were performed in small plastic cell cups (Nicot system[®], Karl Jenter, Metzingen) which were embedded in glass Petri dishes with bees wax (Figure 1) in order to guarantee constant temperatures at the bottom of the cell cups. All tests were carried out at temperatures of 28–30 °C provided by a hotplate. Bees wax has a low thermal conductivity and will therefore compensate for small temperature variations on the surface of the hotplate. The Petri dishes with the bees wax were applied to the hotplate 30 min prior to the experiments in order to ensure a stable temperature. At the beginning of each test series, the temperature within the cell cups was checked by means of an infrared thermometer (Voltcraft IR-1001A, Conrad Electronic SE).

For each test, an adult male mite was introduced into the cell cup together with different female mite stages. A cover glass on the top prevented the mites from escaping from the arena. The male responses toward the female stages were categorized as follows: (1) movement towards/around the female and palpating the female, (2) mounting the female's dorsum (Figure 2 left), and (3) copulation attempts on the female's venter. The duration of each of these behaviors was observed and recorded online over a period of 10 min, using the Observer 2.0 software (Noldus Information Technology).

2.3. Choice tests with different female stages

In a first set of choice tests, male mites were exposed at the same time to an early-stage female



Figure 1. Plastic queen cell cups (Nicot system[®], Karl Jenter, Metzingen) were embedded in a glass Petri dish with beeswax and used for mating bioassays.

deutochrysalis, a freshly molted female, and a mother mite for 10 min. The tests were performed with living as well as with dead female mites. These female mites were killed by 45-min deep freezing and were warmed up prior to the tests.

2.4. Choice tests with female mites before and after molting

In order to identify the most attractive female stages more precisely, we tested female mites at the beginning of the adult molt, directly after the molt, and 24 h after the molt. We therefore collected female deutochrysalis shortly before molting and kept them together with the host pupa and mother mite in the incubator at 31 °C and approximately 60 % humidity. The development stages of the deutochrysalis were checked at 30-min intervals. As soon as the molting started, they were transferred into queen cell cups containing a male, and the male behavior was recorded for 10 min. Subsequently, the male was removed, and the further course of molting was observed under the binocular microscope. As soon as the nymphal skin had been shed by the mites, the freshly molted female was offered again to another new male, and the male's behavior was recorded for 10 min. After that, the freshly molted female was kept in the incubator with a host pupa and a mother mite for 24 h, and then once again presented to a male mite for 10 min. Each of the three female stages was tested against an unattractive control. In the former test series (Section 2.3; Figure 3), the early-stage deutochrysalis was proven to be completely unattractive to males and was therefore used as a control.

2.5. Data analysis

For all data analyses, the SPSS 15.0 statistics software was used. The nonparametric Kruskal–Wallis test was performed to evaluate the male behavior toward different female stages. Differences between groups with $P < 0.05$ were considered as being statistically significant.

3. RESULTS

3.1. Observation of the mating behavior with our bioassay

With this bioassay, the distinct behaviors of the male mites could be clearly distinguished

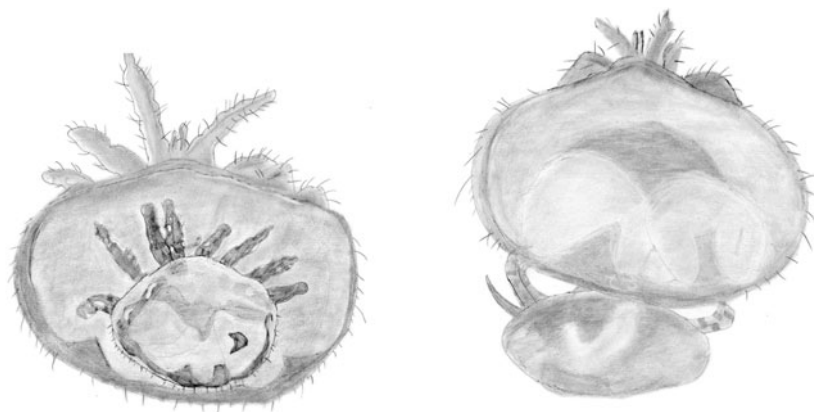


Figure 2. Typical steps of the mating behavior of *Varroa* males before the transfer of the spermatophore: mounting of the female's dorsum (*left*) and movement to the venter (*right*). For details, see text.

when attractive females were offered. The course of mating can be described as a behavioral cascade with four steps: (1) attraction, (2) mounting the female dorsum (Figure 2 left), (3) movement to the venter (Figure 2 right), and (4) transfer of sperm. (1) The cascade starts with the attraction of the male. As soon as the male mite is close to a young female mite, the male follows the female and touches it with the forelegs. (2) Then, the male ascends the female's dorsum and examines the frontal margin of the dorsum with tarsi I, especially at the area of coxae III and IV. Next, the male slips sideways, or from the posterior part of the opisthosoma to the ventral side. (3) In the venter-to-venter position, the male probes the area around the solenostomes, alternating from one side to the other. (4) Then, the male pauses on one side, probably secreting and afterwards transferring the spermatophore into the solenostomes. A short video sequence of the first three steps of this mating behavior is available online (please see [Electronic supplementary material](#)).

3.2. Choice tests with different female stages

Male mites showed positive responses towards both living and dead females. In tests with living females (Figure 3a), males made preferably contact with young females. Males

spent significantly more time walking around young females than walking around old females, whereas differences with regard to deutochrysalis were not significant. After the first contact with forelegs, males mounted the young females in most cases and palpated the dorsum. This mounting behavior towards young females was highly significant if compared to deutochrysalis and old females. Copulation attempts of the males were almost exclusively performed with young females (Kruskal–Wallis, $P < 0.001$, $n = 12$; Figure 3a).

When dead females were offered to male mites, similar behavioral preferences of the males (Figure 3b) could be observed. Furthermore, in these tests the males showed the significant longest mounting behavior (Kruskal–Wallis, $P < 0.05$, $n = 24$) as well as significant longer copulation attempts towards young females (Kruskal–Wallis, $P < 0.001$, $n = 24$). Surprisingly, the male mites showed quite a number of copulation attempts with dead old females (Figure 3a) which was not the case in the test series with living females (Figure 3b).

3.3. Choice tests with female mites before and after molting

Description of the female's molt The course of the female ecdysis proceeds in three major

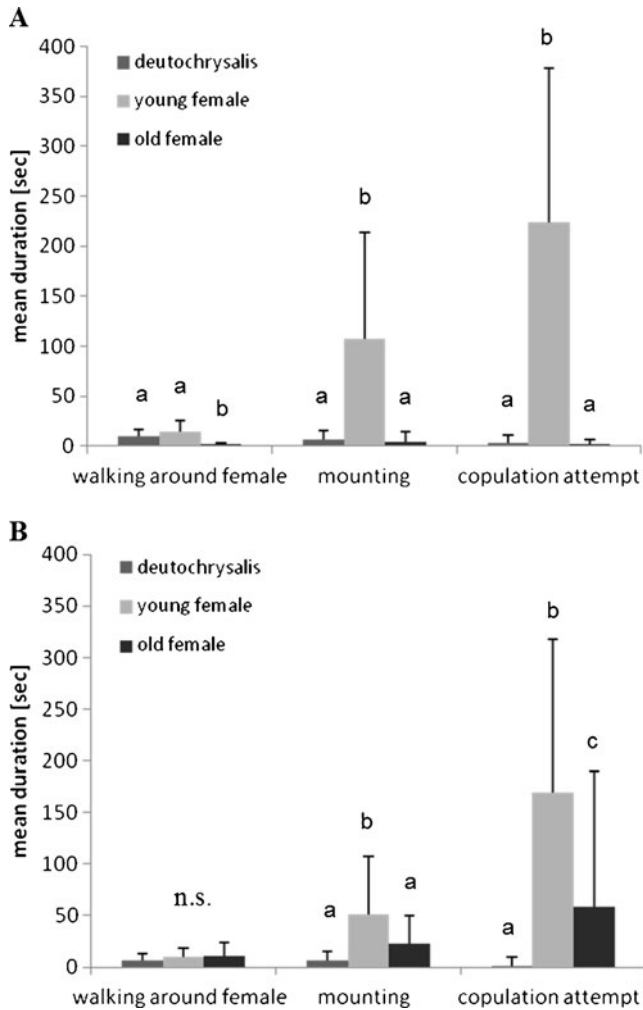


Figure 3. Male responses to different female stages. Female deutochrysalis, freshly molted young females, and old females were offered **a** alive ($n=12$) and **b** freeze-killed ($n=24$) to males. Means followed by *different letters* are significantly different (Kruskal–Wallis test, $P<0.05$).

steps which can be easily distinguished macroscopically: (1) molt of the dorsum, (2) molt of the posterior part of the venter, and (3) molt of the anterior part of the venter including the legs. Shortly before molting, the deutochrysalis starts to move its legs. The first pair of legs is still stretched out forward, the other legs lie under the body, and the deutochrysalis raises the anterior part of the body (Figure 4a). The initial crack appears at

the anterodorsal margin of the opisthosoma. By alternating left–right movements, the integumentary shell is pulled to the posterior margin (Figure 4b) and finally to the ventral side. In doing so, the old skin is folded at the posterior end of the venter between the anal shield and coxae 4 (Figure 4c). At this stage, the old skin has already peeled off the posterior part of the venter. By moving the legs back and forth, the female finally extri-

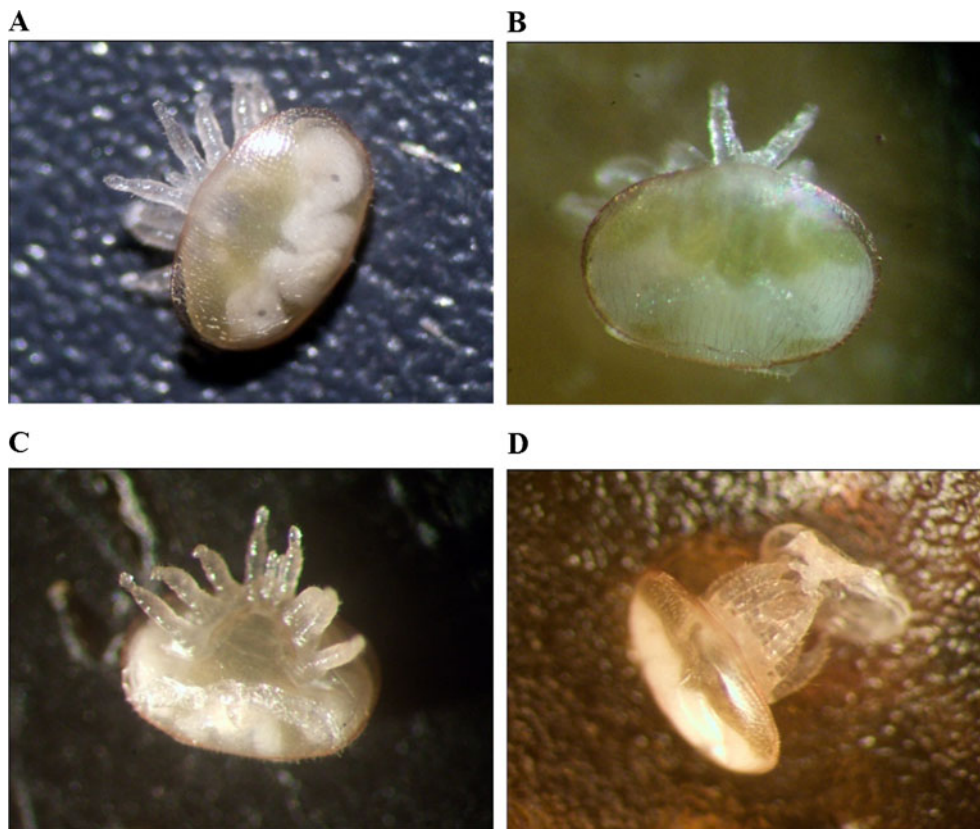


Figure 4. Female development stages during and after the adult molt. At the beginning of molting, the deutochrysalis' first pair of legs is stretched out forward, and the other legs are under the body (**a**, **b**). By alternating left–right movements, the nymphal skin is pulled from the anterior to the posterior margin (**b**) to the ventral side where it is folded between the anal shield and coxae 4 (**c**). Finally, the mite extricates its legs and the anterior part of the venter (including mouthparts) from the nymphal skin (**d**).

cates its legs and the anterior part of the venter (including mouthparts) from the nymphal skin (Figure 4d).

In tests with deutochrysalis at the beginning of the molt (Figure 5a), male contacts were rare, and only a few short copulation attempts could be observed. Compared to the completely unattractive control, the differences were above the significance threshold (mounting, Kruskal–Wallis, $P=0.01$, $n=18$; copulation attempt, Kruskal–Wallis, $P=0.008$, $n=18$). However, shortly after the adult molt, the females became highly attractive to the males. Males showed the whole mating cascade and spent on average more than two thirds of the

observation time with copulation attempts with these freshly molted females (Figure 5b), whereas there was no mating behavior observed towards the control. Differences concerning the mounting behavior and the copulation attempts were highly significant (Kruskal–Wallis, $P<0.001$, $n=18$). When these females were offered again to males 24 h after the molt, a clear decrease in the male copulatory responses was observed. The males spent now less than one third of the observation time with copulation attempts (Figure 5c). However, differences concerning mounting events and copulation attempts were still highly significant (Kruskal–Wallis, $P<0.001$, $n=18$).

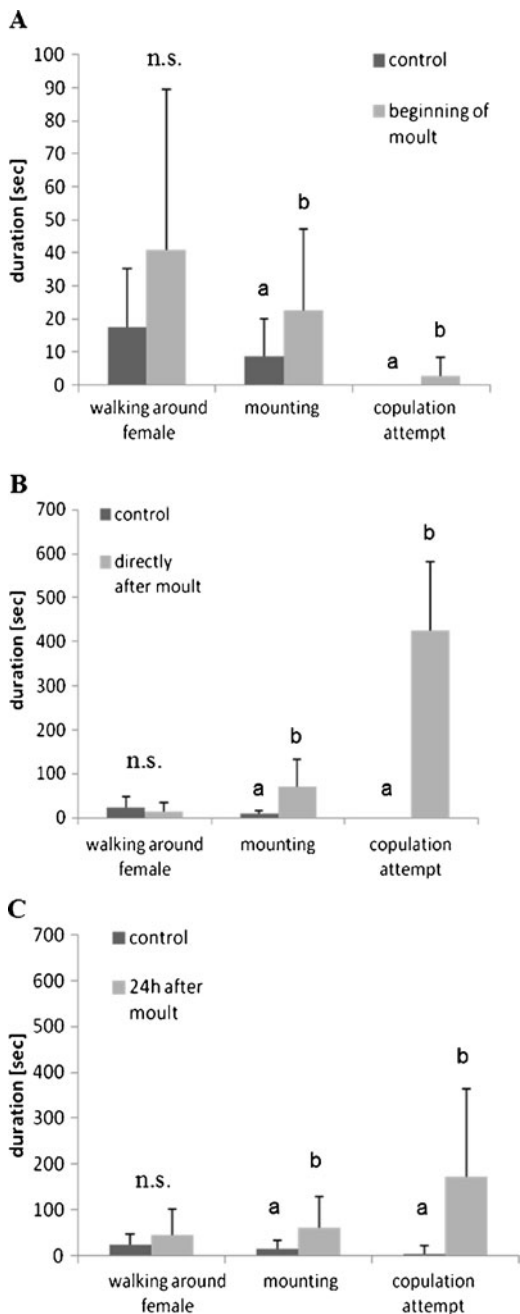


Figure 5. Male responses to females during and shortly after adult molt. Females shortly before (a) and after molting (b) as well as females 24 h after molt (c) were offered to males in three trials, together with white deutochrysalis serving as controls. Means followed by different letters are significantly different (Kruskal–Wallis test, $P < 0.05$, $n = 18$). Due to the low numbers of male responses, different scaling was used in a.

4. DISCUSSION

With this new mating bioassay, we have established a simple setup that allows a standardized quantification of the male mites' mating behavior under laboratory conditions. The setup of the bioassay is easy to be carried out and inexpensive. The wax and Petri dishes can be used during the entire trial period, and only the cell cups have to be replaced. Although the experimental conditions were artificial, no time for acclimatization was necessary. Both male and female mites showed a high vitality during the tests. They moved inside the cell cups very fast and typically used only legs II–IV for walking. The first pair of legs was continuously waved like antennae for probing the environment. Most males began mating as soon as they encountered an attractive female; and the mating behavior observed within this bioassay resembles the one described by Donzé et al. (1996). However, in all test series there were some males that did not show copulation attempts, which led to partially high standard deviations. Such unmotivated male mites might represent males that were either too young (Häußermann, unpublished data) or too old, or that had copulated within the brood cell just before being introduced into the bioassay.

In conclusion, this bioassay provides a possibility for observing and quantifying the mating behavior of mites in the laboratory with reproducible results, and also a basis for the investigation of the involved cues.

In choice tests with living and dead females, males mated preferably with young, freshly molted females regardless of whether the females were alive or dead. The deutochrysalis—living or dead—were completely unattractive to male mites. We hardly ever observed copulation attempts with living old female mites. The freeze-killed old female mites still revealed a certain attractiveness to males, but this was clearly lower compared with young female mites. So far, we do not have a conclusive explanation for the slight-

ly higher attractiveness of dead old females compared to living old female mites. Possibly, old females produce a repellent toward males, which is degraded rapidly when the mite is dead. Old female mites are also more active compared to the young ones, and might therefore hamper undisturbed contacts between males and females. However, this result should first be confirmed by a comparison of both, dead and living females, within the same bioassay.

In order to evaluate more precisely the time period, in which female mites are attractive to males, we tested females prior to and after the adult molt. The results clearly show that female mites become attractive with the adult molt. Shortly before the adult molt of the female mites, the mounting events increased, and even a few copulation attempts could be observed. However, the duration of these copulation attempts was extremely short, indicating that female mites of this stage had not yet reached their full attractiveness to the males. If we offered females immediately after molting, all male mites in our bioassays started with long-term copulation attempts. We assume that the responsible female cues are already present in the late deutochrysalis stage but are not fully available until the female has molted and becomes receptive. It is likely that chemical cues, such as pheromones, are involved in this process, and that they are released directly after the molt, possibly from the now uncovered gonopores. Within the following 24 h, the attractiveness of young adult mites clearly decreases. Although males still make definite copulation attempts towards these females, the differences between freshly molted and 24-h-old females are obvious. Concerning brood cell conditions and the pressure of time for mating, it is reasonable to assume that females are only highly attractive to males within a limited time span. As the female mites' attractiveness is directly related to the adult molt, males are able to recognize these females immediately and can start mating as

soon as the female becomes sexually mature. This is essential as repeated matings are necessary for the successful transfer of a sufficient number of spermatozoa (Donzé et al. 1996). As long as there are no younger females available, males continue with the mating process. However, the willingness to mate with older females decreases, and already 24 h after the molt, the females are clearly less attractive. Thus, males are obviously able to distinguish between freshly molted and older females and mate with the youngest mature females.

The mating of the young female mites within the sealed brood cells represents a crucial phase of the reproductive cycle of *V. destructor*. Female eggs are laid at 30-h intervals, and therefore, young adult female mites appear at similar intervals. Depending on the capping period of the brood cells, only one to three adult female mites can reach maturity (Rosenkranz et al. 2010). Additionally, successful mating seems to be a prerequisite for the survival of the daughter mites because all phoretic mites on the adult bees contain sperm within their spermatheca (Garrido and Rosenkranz 2003; Weller 2008; Frey and Rosenkranz, unpublished data). As mating can only occur within the capped brood cell, the time period for the mating process is very limited. Therefore, one can assume a strong selection pressure on mechanisms securing that (1) adult female mites are mated as soon as possible (= directly after molting), and (2) that the male is able to select freshly molted (unmated) females from older ones in order not to "waste time" on females having already been mated. Our results clearly indicate that freshly molted females are most attractive to males, and that this attractiveness is presumably based on a female sex pheromone being released during the process of molting. Older females lose at least in part their attractiveness by yet unknown mechanisms. These findings should stimulate further research with the objective of disturbing the male's mating behavior as a first approach for a biological *Varroa* control.

ACKNOWLEDGMENTS

We thank the Montagu Foundation and the Agroscope Liebefeld-Posieux for providing financial support. We are grateful to Barbara Locke for critical reading of the manuscript. Last but not least, we express our cordial thanks to Helen Kilian-Rosenkranz for the drawings in Figure 2.

Le comportement d'accouplement de *Varroa destructor* est déclenché par une phéromone sexuelle de la femelle. Partie 1: Comportement préférentiel des acariens mâles dans un essai biologique en laboratoire

Varroa destructor / comportement d'accouplement / comportement sexuel / mâle / acarien / observation en laboratoire

Das Paarungsverhalten von *Varroa destructor* wird durch ein weibliches Sexualpheromon gesteuert. Teil 1: Präferenzverhalten der männlichen Milben in einem Labor-Biotest

Varroa destructor / Paarungsverhalten / Honigbiene / Biotest

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