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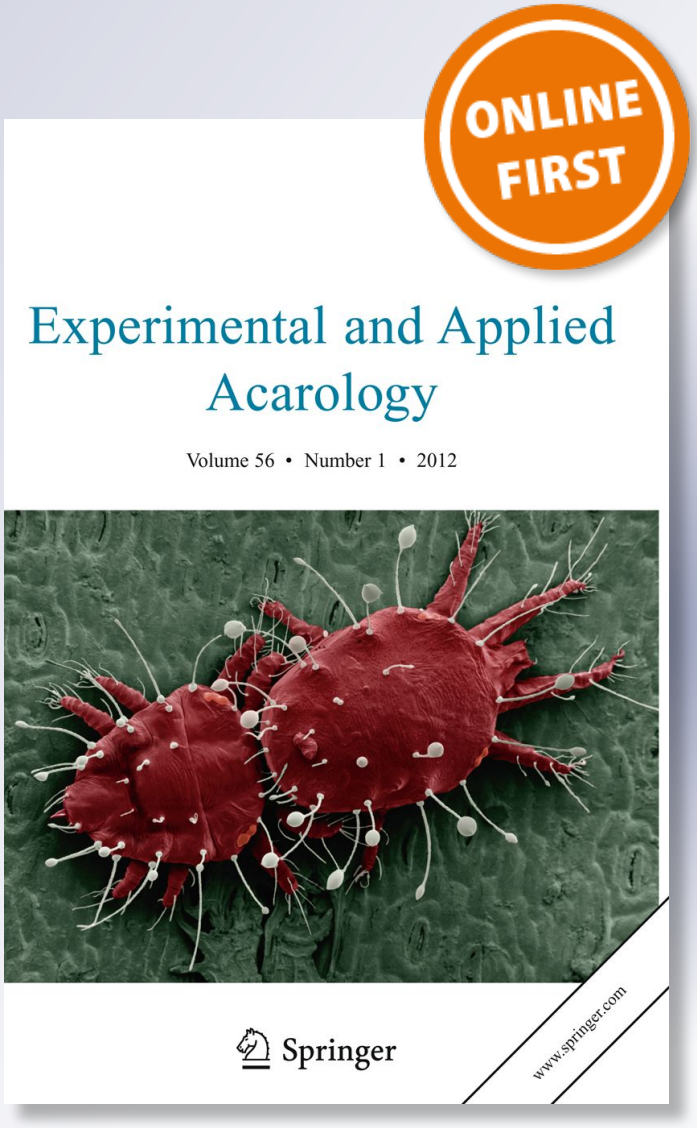

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Age and reproductive status of adult *Varroa* mites affect grooming success of honey bees

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Abstract This study evaluated for the first time the grooming response of honey bees to *Varroa* mites of different ages and reproductive statuses in the laboratory. Plastic cages containing a section of dark comb and about 200 bees were inoculated with groups of four classes of mites: gravid, phoretic foundresses, phoretic daughters and a combination of gravid and phoretic foundress mites. Each cage received 20 mites belonging to one of these classes. Our results showed that, 1 day after mite inoculation, phoretic daughter mites were the most prone to grooming by honey bees with an average mite drop of 49.8 ± 2.6 %. The lowest mite drop was recorded for bees inoculated with phoretic foundresses (30.3 ± 3.6 %) but was comparable to bees inoculated with gravid mites (31.8 ± 3.8 %) and the combination of gravid and phoretic foundress mites (34.2 ± 3.2 %). No differences among mite types were detected during the second and third days of observation. Regardless of mite type, the highest mite drop was recorded on the first day (35 ± 2.1 %) compared to the drop for any subsequent day (<10 %). Because of the great reproductive potential of daughter mites, their inclusion in assessments of grooming behaviour may increase our insight into the importance of grooming in mite resistance.

Keywords *Varroa destructor* · Grooming · Hygienic behaviour · Gravid mites · Daughter mites

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Introduction

Grooming and hygienic behaviours are two of the traits employed by honey bees in order to remove the parasitic mite *Varroa destructor* from nests. Together, both behaviours target the mite during both their phoretic and reproductive stages. No study, however, has investigated whether or not there is any link between the expressions of these behaviours.

The morphology of the *Varroa* mite is central to its ability to attach to the honey bee host. Rath (1999) observed that the mite's concave ventral and convex dorsal sides are of "foremost" importance in enabling it to adhere to the bee or hide between abdominal sternites. Equipped with a sclerotized carapace that protects the mite, and setae that assist in adhering to the bee's body, the mite is well adapted to its phoretic period when attached to the host (Rosenkranz et al. 2010).

Hygienic behaviour is reported to occur predominantly 3–5 days post-capping (Harris 2007). This observation means that the foundress mite will not have finished her reproductive cycle before being removed from the cell, and in many cases she may have laid only one egg. In a gravid foundress, the mature egg reaches a considerable size and occupies much of her opisthosoma (Akimov et al. 1988). Further, the membrane between the sternal shields stretches and the genito-ventral shield protrudes (Akimov and Yastrebstov 1984). In contrast, the opisthosoma of a phoretic mite is not swollen because it is usually in a previtellogenic stage; oogenesis only being activated upon entering the cell (Garrido et al. 2000).

Similarly, the body of a newly emerged daughter mite is not well sclerotized. Having a softer carapace and setae may negatively affect these mites's phoretic ability, thereby facilitating their capture by bees. This is particularly true for the second and third *Varroa* daughters which have only a few hours to 1 day after having matured to adults prior to the emergence of host bees (Martin 1994). In addition, the softer and more tender setae of young daughter mites may influence their locomotory ability.

Several researchers have highlighted the difficulty in measuring grooming behaviour (Rosenkranz et al. 1997; Aumeier 2001). Without reliable measurement, grooming behaviour will always be excluded in any breeding program. It is possible that grooming assays can be improved by using mites with an optimum age and reproductive status. Because of the morphological characteristics of both daughter and gravid mites, we hypothesized that these classes of mites are more susceptible to the grooming behaviour of honey bees than phoretic mites. Therefore, this study compared the removal response of honey bees to gravid mites, which may be released when bees performed hygienic activities, and mature daughter mites that were phoretic on emerging bees.

Materials and methods

Mite and bee sources

Test mites were collected from four highly infested colonies of Italian honey bees maintained at the USDA-ARS Honey Bee Breeding, Genetics and Physiology Laboratory in Baton Rouge, Louisiana, USA. Four classes of mites were assessed: (a) gravid mites, (b) phoretic foundress mites, (c) a combination of gravid and phoretic foundress mites, and (d) phoretic daughter mites. To obtain gravid mites, the queen of each source colony was caged on an empty frame using a push-in cage (length = 42 cm, width = 13 cm, height = 3 cm) for egg-laying. After 9 days, newly sealed brood were opened with a

forceps and gravid mites were collected using an insect brush. At this stage, the foundress mites had initiated oogenesis and were either just about to lay their first egg or already had done so. Phoretic foundress (dark color) and also matured daughter (slightly brown in color) mites were collected by removing frames of emerging brood from source colonies. Also, frames of emerging brood from the source colonies were placed in screen boxes inside an incubator (35 °C) overnight for bees to emerge. The following day, newly emerged bees were brushed off the frames into an arena made of a plastic sheet placed on top of a white sheet of paper. Each bee was then examined for the presence of mites. Phoretic foundress and daughter mites, differentiated by the degree of sclerotization of the carapace when viewed under a dissecting microscope, were then removed from the bees using an insect brush. Also, the use of mites that were phoretic on the newly emerged bees (rather than opening sealed brood) ensured collection of matured daughter mites. Mites were then inoculated immediately onto the caged bees using an insect brush.

Worker bees subject to this grooming behaviour study were obtained from two honey bee colonies with the lowest *Varroa* infestations (colony 1 = 0 %; colony 2 = 0.2 % adult infestations) in the apiary. From each colony, worker bees from two brood and two honey frames were shaken into a box. This method ensured collection of a representative sample of different ages of worker bees in the hive. Worker bees were then scooped using a measuring cup (1/4 cup), which collected an average of 202 ± 25 bees and placed into each of the plastic cages.

Inoculation of caged bees

The cages used in this study were similar to those of Webster (1994) with some modifications (Fig. 1). Each cage was made of two plastic cups (basal diameter = 6 cm; mouth diameter = 10 cm), one nested inside the other. The base of the inner cup was replaced with a circular screen (1 × 1 mm). Before installation of bees, a small section of dark comb (4 × 4 cm) was affixed at the middle of the screen to provide a place for the bees to cluster. The base of the outside cup had two holes (diameter = 3 cm) that received two scintillation vials which rested on the screen closure of the inner cup; one contained sugar syrup (1:1, sucrose:water) and one water. Each of the cups had a small hole (diameter = 1.5 cm) on the side about 4 cm above the cup's mouth. The outer cup was rotated to align the two holes for easy mite introduction and also removal of dead bees. The mouth of each inner cup was also closed with a circular mesh (1 × 1 mm) that prevented bees from escaping but allowed mites to pass through. Eight small triangular (base = 1 cm; height = 1 cm) notches were also cut around the mouth of each cup. The screen and the notches served as ventilation for the bees. Each cage sat on a petri dish that had been smeared with a mixture of petroleum jelly and vegetable oil (1:1) to trap mites that dropped.

A total of 54 cages were established. There were 22 cages (one cage = one replicate) containing gravid mites, 12 containing phoretic foundress mites, 9 containing phoretic daughter mites and 11 containing the combination (gravid and phoretic) treatment. Each cage received 20 mites. For the combination group, each cage received 10 gravid and 10 phoretic foundress mites. The mites were inoculated onto a bee using an insect brush through the small hole at the side of each cup. When the brush came into contact with a bee, the mite readily transferred onto the host. As mites may have become disoriented or injured during mite collection, any mite that did not successfully attach to a host bee after a second attempt was discarded. The cages were placed randomly on trays in an incubator (35 °C). A pan of water was also provided inside the incubator to prevent desiccation. Each

Fig. 1 The modified plastic cages with cohorts of bees inoculated with mites

petri dish was examined for the presence or absence of mites that dropped every day. Collected mites were counted and then individually inspected under a dissecting microscope for the presence or absence of injuries. In the combination group, mites were examined for gravidness (swollen opisthosoma). Also, all dead bees at the bottom of the cups were removed and examined for the presence of mites. If a dead bee was infested, mites were inoculated back onto live caged bees. At the end of the experiment, the bees were frozen and then washed using soapy water (Rinderer et al. 2004) to determine the number of mites remaining phoretic on the bees.

Three trials were conducted: the first trial used only gravid mites and test bees from one colony while the second and third trials evaluated all four treatments using workers from two colonies. The first trial was monitored for 1 week. However, it was noted that there was very little change in the number of mites that were removed after 3 days. Hence, data for the first 3 days only were considered for analysis. Trials 2 and 3 were consequently conducted for 3 days.

Data analysis

Since some mites were not accounted for after the experiment, mite drop was calculated as the percentage of mites that fell out of the total number of mites recovered (mites that dropped plus mites that remained on the bees). The percentages were transformed using an arcsine square root transformation before analysis to approximate normality. A complete randomized block design analysis of variance (ANOVA) was used with mite type and observation time modelled as fixed effects and trial as a random block effect. Means were separated with a post hoc SLICE test in SAS (SAS version 9.2, SAS Institute 2008).

Results

For the proportion of mites that dropped, ANOVA revealed no significant differences among treatment groups or mite types ($F = 2.01$; df $= 3$; $P = 0.12$). However, a significant interaction between mite type and time of observation ($F = 3.61$; df $= 6$; $P = 0.003$) was detected (Fig. 2). One day after mite inoculation, mite drop varied among mite types ($F = 6.44$; df $= 3$; $P = 0.001$). The highest mite drop was recorded in bees inoculated with daughter mites with the lowest mite drop observed in bees inoculated with phoretic foundress mites which was comparable to those bees inoculated with gravid and a combination of gravid and phoretic foundress mites. No differences among mite types were detected on the second ($F = 1.21$; f $= 3$; $P = 0.31$) and third day ($F = 1.56$; df $= 3$; $P = 0.20$) of observation. Regardless of mite type, the highest mite drop was recorded one day after mite inoculation (mean $= 35 \pm 2.1$ %, n $= 54$, range 11.1–70.6 %) ($F = 123.8$; df $= 2$; $P = 0.001$). Mite drops on day 2 (9.7 ± 1.4 %, n $= 54$, range 0–50 %) and day 3 of observation (5.2 ± 0.6 %, n $= 54$, range 0–16.7 %) were comparatively low. Only one mite with a damaged leg was observed.

Discussion

The results of this study indicate that the grooming response of honey bees may be influenced by the age of mites exposed to this behavior. Mature daughter mites (slightly brown) collected from newly emerged bees were more prone to removal than the foundress phoretic or gravid mites, on the first day after inoculation. The increased removal of daughter mites may be due to their degree of maturity, their bodies being softer due to the lower degree of sclerotization. This softness may have negatively affected their ability to move or securely attach to host bees facilitating their capture by the bees. This decreased mobility may be the reason why a higher proportion of injured light colored mites were observed by Moosbeckhofer (1992). Although the daughter mites used in this study were phoretic on newly emerged bees, the mites may need more than 1 day post bees' emergence for increased body sclerotization and thus, better mobility.

The removal of daughter mites (about 61 % in this study) from honey bee colonies may have a substantial impact on the growth of *Varroa* mite populations. In general, mating occurs just after the deutonymphs moult into adults (Donzé et al. 1996). Also, worker brood can allow the development of two mated daughter mites (Lobb and Martin 1997).

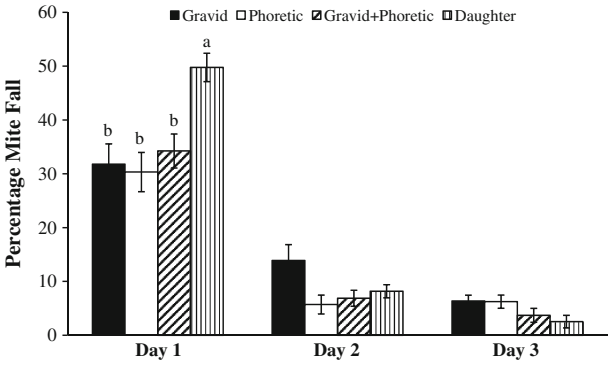

Fig. 2 Mean percentages of mite drop in cages with honey bees inoculated with *Varroa* mites of different ages or reproductive status through time. For each time of observation, *bars* with *different letters* are significantly different at $P < 0.05$

Having been collected from newly emerged bees, the mature daughters in this study would have mated within the natal cells. Further, hygienic behaviour can occur at all stages of brood development (Harris et al. 2010). The hygienic removal of tan-bodied pupae or older will allow the release of the first mated daughter mites. These daughter mites are able to immediately invade suitable hosts. Lobb and Martin (1997) documented that even pale-colored and slightly brown mites that were collected from bottom boards of colonies with emerging brood had spermatozoa in their spermathecae and reproduced (23 and 44 %, respectively) when inoculated into newly sealed larvae. These observations suggest that young mites do not have to be phoretic on adult bees after emergence prior to reproducing. Although the population growth of mites in a colony suggests that it is not common for newly emerged daughters to skip the phoretic stage, doing so would allow mites to produce more generations per year.

The low removal of gravid mites suggests that this group of mites were able to avoid grooming by the bees. Normally, gravid mites have a swollen opisthosoma due to the presence of a matured egg that occupies much of the opisthosoma (Akimov and Yastrebstov 1984). We hypothesized that this condition would make gravid mites more prone to being removed by grooming. However, this was not the case. We observed that the swelling of the abdomen of gravid mites was reduced 1 day after mite inoculation. This reduction of gravidness may have enabled the mites to avoid being groomed. The decrease in swelling may be the result of expulsion of the egg from the genital opening since some mites that dropped on the petri dish had eggs on their genito-ventral shield. Oosorption, or when vitellogenesis ceases and yolked oocytes degenerate (Bell and Bohm 1975), may also explain the reduced swelling of the mites' opisthosoma.

We also observed that most mites were groomed on the first day following inoculation rather than in subsequent days. Detection of the mites by the bees may be due to foreign scent (Rosenkranz et al. 1993) or cuticular hydrocarbon (Nation et al. 1992) being different from the test bees receiving the inoculum mites. However, in each trial all test mites came from the same source colony and thus, should have been equally regarded as foreign by the bees, thereby controlling for this effect. Our observation agreed with the findings of earlier studies indicating high removal of young resident mites (Boot et al. 1995, Lobb and Martin 1997). It is also possible that the decreased mite removal through time was due to increasing maturity of the bodies of the daughter mites. Further, the reduced swelling due to oosorption by gravid mites may explain the significant decrease in the proportion of mites that dropped on the second and third day of observation. For the phoretic foundress mites, increased removal on the first day may be due to their idiosomal shape. While examining mites from newly emerged bees under a dissecting microscope, the phoretic foundress did not have the "concave" appearance of their ventral idiosoma which is a characteristic of long-time phoretic mites. Rather, the mites had "flat" idiosoma, but not as swollen as those of the gravid mites. Being fresh from the cells, it is possible that the phoretic foundress mites may still have a full midgut, which is known to occupy about 2/3 of the volume of *Varroa*'s body cavity (Akimov et al. 1988).

It is also possible that the results of this experiment are partly an artefact of protocol i.e., inoculation of live mites onto bees. According to Rath (1999), bees are disturbed by mites and therefore prompted to groom only when the mites have left "phoretically safe positions" on the host or have just arrived on the body. This experiment therefore mimicked what would occur when mites become phoretic on a new host and the results support the statement by Rath. It indicates that in nature, as these mites emerge with adult bees or are removed due to hygiene, they are highly vulnerable to grooming. The significantly higher number of immature mites groomed off bees across all trials supports a greater

susceptibility of these mites. By collecting the mites that failed to attach directly after their introduction and then re-inoculating them, we sought to minimise the effect of protocol.

It is also interesting to note that only one injured mite out of 1,080 inoculum mites was observed during the experiment. This observation agrees well with the results of several grooming behaviour studies wherein very few damaged *Varroa* mites were reported (Büchler et al. 1992; Aumeier 2001). Although no outward signs of injury are noticeable, internal damages may affect the well-being of the mites. Aumeier (2001) found that even in cases where mites were thought to have been caught by bees' mandibles, no visible signs of damage to their bodies were observed. A similar observation was reported by Khongphinitbunjong et al. (2012) regarding *Tropilaelaps mercedesae* wherein a mite observed to have been caught by the mandibles of a bee did not have obvious injury but was unable to walk and died after a few hours. Reproductive success in undamaged mites removed by grooming may also be reduced (Fries et al. 1996).

In conclusion, daughter and gravid mites have great reproductive potential when suitable hosts are available. Their removal from colonies may have a strong negative impact on overall mite population growth. Bienefeld et al. (1999) recommended that "exclusive consideration" be given to the extent of damage to adult mites in grooming behaviour assays. However, assays that include daughter mites (injured or not) which are removed from the nest may reveal a more concrete contribution of grooming to the overall resistance of bees to *Varroa* mites.

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