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Behavior of varroa mites in worker brood cells of Africanized honey bees

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Abstract The ectoparasitic mite *Varroa destructor* is currently the most important pest of the honey bee, *Apis mellifera*. Because mite reproduction occurs within the sealed cell, the direct observation of varroa activity inside the cell is difficult. A video observation method using transparent polystyrol cells containing infested brood was used to analyze the behavior of varroa mites in worker brood of Africanized honey bees. We recorded how mites feed on the larva and pupa, construct a fecal accumulation site and how the bee larva carried out some longitudinal movements around the cell. The feeding activity of the foundress mite varies during the course of the cycle. On the prepupa mites were found to feed often (0.3 ± 0.2 bouts h^{-1}) for a period of 8.7 ± 8.4 min h^{-1} and there was no preference for a specific segment as feeding site. On the opposite, during the pupal stage mites fed less often (0.1 ± 0.1 bouts h^{-1}) for a period of 6.2 ± 4.0 min h^{-1} and almost always at a particular site (92.4%). On pupa, 83.7% of the feeding was on the 2nd abdominal segment ($n = 92$), and only few perforations were found on the thorax. Varroa shows a preference for defecation in the posterior part of the cell (cell apex), close to the bee′s anal zone. We found a high correlation between the position of the feeding site on the pupa and the position of the fecal accumulation on the cell wall. Most infested cells have only one fecal accumulation site and it was the favorite resting site for the mite, where it spent 24.3 ± 3.9 min h^{-1}. Longitudinal displacements were observed in 28.0% ($n = 25$) of the analyzed bee larvae. Turning movements around the cell, from the bottom to the top, were carried out by these larvae, mainly during the second day (47.7 ± 22.5 min h^{-1}), just before pupation, with a total time of 874.9 ± 262.2 min day^{-1} ($n = 7$ individuals). These results in worker brood of Africanized bees demonstrate adaptations of varroa mites to parasitizing the developing bee inside the capped brood cells.

Keywords *Varroa destructor* · Capped artificial cells · Feeding behavior · Fecal accumulation site · Displacement behavior · Feeding site · Africanized honey bees

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Introduction

The ectoparasitic mite *Varroa destructor* is currently the most important pest of the honey bee *Apis mellifera* (Bailey and Ball 1991). Many reports exist of severe damage and loss of thousands of honey bee colonies caused by infestation with varroa mites, especially in temperate areas of Europe and Asia (De Jong et al. 1982; De Jong 1997).

Varroa mites live apparently in a longtime association with their natural host *A. cerana*, the Indian honey bee, in South East Asia. Serious damage to these bees has never been reported, and treatment against varroa is not needed in beekeeping with *A. cerana* (Büchler 1994; Boot et al. 1997). Nevertheless, since its first contact with *A. mellifera*, it can be seen that the population dynamics of the mite varies from one region to another. Varroa is the most important pest of European races of the western honey bee. Colonies infested by varroa mites are damaged and are likely to die over winter only 3 years post infestation (Korpela et al. 1993). In other regions, Brazil among them, varroa was introduced more than 30 years ago and established itself at low levels of infestation, without causing apparent damage to apiculture with Africanized honey bees (AHB) (De Jong 1997; Moretto and Leonidas 2001, 2003).

Reproduction of varroa mites takes place only within the sealed brood cells of honey bees, during bee development (Ifantidis and Rosenkranz 1988; Erickson et al. 1994). Varroa females initiate reproduction by entering the brood cells of last-stage worker or drone larvae, normally around 20 or 40 h, respectively, before the cell is sealed. After leaving the larval food on the bottom of the sealed cell, the mites begin feeding on the haemolymph of the prepupa (Donzé and Guerin 1994). About 60–70 h after the brood cell is sealed, the first egg is laid. They are laid one by one, usually on the cell wall, at intervals of about 30 h (Martin 1994).

At first sight the brood cell would appear to provide an ideal environment for mite reproduction, with its stable temperature and humidity, accessible food source, and absence of predators or competitor species (Donzé and Guerin 1994). However, the time available for mite reproduction is limited and the changes occasioned by the bee′s development limit the space available for the foundress mite and its descendants. Furthermore, physiological characteristics of the honey bee larvae or pupae might also afford some resistance to varroa mites. For example, mite reproductive rates may be lowered if there is reduced feeding activity on the larvae or pupae by the foundress mites (Grandi-Hoffman et al. 2002) or offspring mortality due to the bee′s movement at pupation can occur. Indeed, traits expressed by bee larva and pupa might be in part responsible for the lower fertility rates of mites in the brood of Africanized bees in Brazil, which show an increased tolerance to *V. destructor* (Ritter and De Jong 1984). Nevertheless, recent studies on AHB colonies from Brazil by Garrido et al. (2003) revealed that mite fertility in singly infested worker brood cells increased to 82%, whereas previous studies had demonstrated a 50% average mite fertility. Furthermore, Carneiro et al. (2007) indicated that the percentage of fertile mites in worker brood of AHB in Brazil increased from 56% in the 1980s to 86% in 2005–2006.

Because mite reproduction occurs within the sealed cell, the direct observation of varroa activity inside the cell is difficult. Observations on transparent cells in European bees demonstrate remarkable adaptations on behalf of varroa in parasitizing the developing bee within the brood cells (Donzé and Guerin 1994, 1997; Donzé et al. 1998). In addition, observations (video filmed) of mites sticking in the food without motion, have also been made (A. de Ruijter, pers. comm., 2003). It is reported that mites sticking in the food could start feeding too late for the oviposition trigger or even may be trapped between the cell wall and the cocoon and die (Martin 1995).

In worker brood cells of AHB, factors that limited varroa reproduction ability included non-fertile female mites, mortality of emerging mites, and foundresses that produced only immature stages (Calderón et al. 2003; Correa-Marquez et al. 2003; Mondragon et al. 2006). Because reduced reproduction of varroa is regarded as the most important factor in tolerance of Africanized bees towards this parasite, an observation method using artificial cells was used to analyze the behavior of varroa mites in worker brood cells of AHB. So far no experiments under such well-defined conditions have been carried out with Africanized bees. One approach to controlling varroa mites would be to select bees that suppress or delay mite reproduction. All mite offspring that have not reached adult stage have no chance of survival when the new adult bee emerges from the cell (Boecking and Ritter 1993).

Materials and methods

The study was carried out at CINAT of the Universidad Nacional, Heredia, Costa Rica (10°01′N, 84°07′W; 1,130 m altitude). The experiments were conducted from January to December 2006 using four AHB colonies. Electrophoretic analysis of two enzymes—hexokinase (HK) and malatedehydrogenase (MDH)—by the use of polyacrilamide gels was done to analyze the degree of africanization in experimental colonies. Varroa detected in Costa Rica was confirmed to be the Russian haplotype (De Guzman, pers. comm., 1999); this form is also referred to as the Korean haplotype (Anderson and Trueman 2000). An observation method using artificial cells was used to analyze the behavior of varroa mites in worker brood. From video observations on transparent polystyrol cells containing infested brood, we set out to establish how the female succeeds in parasitizing the developing bee, feeds on the larvae, and constructs a fecal accumulation site. Five non-infested cells were also analyzed for larvae and pupae development.

Artificial brood cells

In order to analyze varroa behavior in worker brood of AHB, artificial cells containing naturally reared brood, infested by a single mite were used for continuous observation. Cylindrical transparent polystyrol cells with internal dimensions of 5.1 mm diameter × 14 mm long (European cell size) for workers were used. The dimensions of the polystyrol cells are similar to those of natural ones and those cited in the literature for workers. These artificial cells were inserted into a brood comb and put into an experimental colony. The position of the artificial cells in the comb was recorded on transparent sheets. We covered the cells with parafilm to protect them from wax and added some honey to induce bees to prepare them for oviposition. The cells were checked every day for oviposition. About 8 days after oviposition, the time of sealing of each cell was recorded at short intervals ($\sim$4 h).

The infested cells were transferred to a laboratory incubator (water jacketed) maintained at 35°C and 60% RH (simulating hive conditions of temperature and humidity). Considering the importance of geotaxis in varroa (Donzé and Guerin 1994), the cells were placed in the natural position (pupa on its back) fixed to a piece of cardboard and turned only occasionally for observations.

Direct observations of the cells were made with a micro-camera connected to a time-lapse VHS and recorded a minimum of 6 days (144 h) and a maximum of 8 days (192 h) (about 2 frames s^{-1}). This system allowed direct observation of the cell, as well as

recording of varroa behavior. A red light source illuminated the cell. Only one artificial cell was recorded at a time.

Observations on brood cells

Based on preliminary observations an ethogram was established and the following behavior was studied for varroa mites: how the mite chooses a site for feeding, feeding frequency, hourly mean amount of feeding, mean duration of feeding bouts, and how varroa initiates and subsequently succeeds to build a fecal accumulation site. In addition, displacement activity of the bee larva (prepupa) was recorded.

A mite was considered to be feeding if it was immobile on the feeding site with the head region inclined and pushed against the cuticle of the bee, showing up and down movement in the anus region. Defecating behavior was reported when varroa abruptly stops, waggles its anus dorsoventrally and deposits the feces. To establish the position of the fecal accumulation site, the artificial cell was divided in three sections (anterior, medium and posterior). The posterior section corresponded with the cell apex and the abdomen of the pupa. To confirm that the position of the feces was not an artifact arising only in the artificial cells, we observed the position of feces in natural cells as well. Inactive periods were considered when the mite was immobile and performed no detectable behavioral activity. Longitudinal displacements (displacement behavior) of the bee larva were registered when the larva turned around in the cell, from the bottom to the top.

After 8 days of observation (192 h after capping) the cells were opened to examine the contents. Bee pupa and mites were taken out, and mites (mature and immature stages) were identified and counted using a stereoscope microscope (10× magnification). The bottom of the cell was also examined. In addition, the exuviae of the bee brood were removed and checked for mites. The mite condition (foundress and offspring) was evaluated using the following parameters: general aspect, locomotion (walking), and appendage motion (legs, chelicerae).

Position of the feeding site on the pupa

Because the number and exact position of the mite-made (for feeding) integumental wounds on the pupal stage are difficult to detect in natural conditions, a vital staining was used (Kanbar and Engels 2004a). For the vital staining, an incubation medium consisting of insect ringer solution adjusted to pH 6.8 was prepared and trypan blue dye was added. Pupa were vital stained at room temperature by incubating them for 30 min into 20 ml of medium, followed by a 3 min rinse in a ringer solution without dye. Controls were incubated in dye-free ringer. All recorded pupae were analyzed for the position of the wound. In addition, naturally infested pupae (of 8 days or more) were also analyzed for the position of the wound.

Time-activity analysis

For each observation, the total duration and the frequency of each behavior displayed on either the bee brood or the cell wall were calculated. To correlate the position of the feeding site on the pupa and the position of the fecal accumulation site on the cell wall, we analyzed the section in the cell (anterior, medium and posterior) where the fecal

accumulation was established and the location of the feeding site (exact position of the feeding site on the pupa was confirmed by the vital staining).

Results

Varroa mite behavior was recorded in 28 artificial worker size cells, but not each cell was used for analysis. In three cells, the bee brood (larvae or pupae) showed disease symptoms. From observations in these cells, we were able to describe how the mite prepares a feeding site on the bee pupa and constructs a fecal accumulation site on the cell wall. Furthermore, how the bee larvae carry out some specific movements around the cell (longitudinal displacement).

Feeding behavior

The feeding activity of the foundress mite varies during the course of the cycle. On the prepupa the mite feeds often (mean ± SE: 0.3 ± 0.2 bouts h^{-1}, $n = 20$ individuals) for a period of 8.7 ± 8.4 min h^{-1} and there was no preference for a specific segment as a feeding site. The foundress mite often changes location for feeding. However, during the pupal stage the mite feeds less often (0.1 ± 0.1 bouts h^{-1}, $n = 20$ individuals) for a period of 6.2 ± 4.0 min h^{-1} and almost always at the same point.

There was a difference between the mean number of times the mite feeds on prepupa (6.5 ± 3.7) and pupa (3.4 ± 1.9) ($\chi^2 = 38.4$, $P < 0.01$, $df = 1$; Fig. 1). On the prepupa the mite feeds 200.5 ± 171.9 min day^{-1} ($n = 20$), whereas on the pupa it feeds 149.1 ± 96.0 min day^{-1} ($n = 20$).

By contrast with the first short feeding bout of 8.4 ± 5.8 min on the prepupa, the first feed by the adult female mite on the pupa last 35.3 ± 23.9 min ($n = 20$) ($\chi^2 = 114.5$, $P < 0.001$, $df = 1$).

Position of the feeding site on the pupal stage

The wounds inflicted on pupae in capped brood cells infested with a single mite, were studied after visualization by vital staining with trypan blue. In the pupal stage of worker brood, the integumental wounds were easily detected: the blue margin of the wound contrasts in particular with the white-brown skin of early pupal stages (6 days). Also in

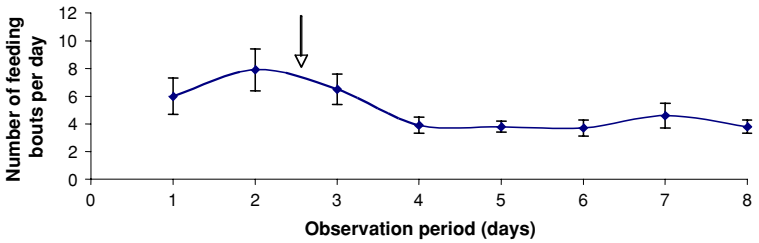

Fig. 1 Feeding bouts of *Varroa destructor* in worker brood cells of Africanized bees during a period of 8 days (192 h) (means ± SE). On the prepupa the mite feeds often. The *arrow* indicates the beginning of the pupal stage

Table 1 Position and amount of varroa-made integumental wounds on the surface of the Africanized bee worker pupal body

Position and amount of wounds	%
Abdomen	83.7
Thorax	16.3
One perforation	92.4
Two perforations	6.5
Three perforations	1.1

later pupal phases (8 days or more), the enlarged puncture was easy to localize as a blue spot.

On pupa a high percentage of the wounds was on the abdomen, specifically on the 2nd abdominal segment and mainly on the left side ($n = 92$). Few perforations were found on segments three to five of the abdomen. On the majority of pupa there was only one perforation (Table 1).

Construction of the fecal accumulation site

Prior to the construction of a fecal accumulation site, the mite walked on the cell wall. On the pupal stage, varroa shows a preference for defecation in the posterior part of the cell (cell apex), usually close to the bee′s anal zone, near the feeding site on the pupa.

Varroa females concentrate the feces on a little spot. Fecal accumulation site formation started with the deposition of a few feces near one another. The accumulation site appears as a bright white amorphous mass that contrasts in color with the transparent cell wall to which it is attached. Although relatively small, ~0.3 mm in diameter, masses of white excreta are easily seen in good light and it is a sign of mite infestation. During the second and fifth day the mites defecate more frequently, five and six times daily, respectively ($n = 20$ cells), decreasing to one time on the seventh day and zero on day eight (Fig. 2). Most of the infested cells have only one fecal accumulation site (64.3%). Two or more defecation sites were found in six cells. No fecal accumulation was found in only four cells.

The fecal accumulation was the favorite resting site for the mite, it spent 24.3 ± 3.9 min h^{-1} at this site. During the second day, the mite stayed the longest at the fecal accumulation site (665.4 ± 337.1 min day^{-1}; $n = 23$ individuals). Behavior of varroa appeared to be in relation to the fecal accumulation and progressively the mite demonstrated the following behavioral routine: varroa descends onto the pupa and accesses the feeding site. After feeding, the mite returns to the cell wall where it stops on the fecal accumulation site, usually to defecate. A significant association was observed between the

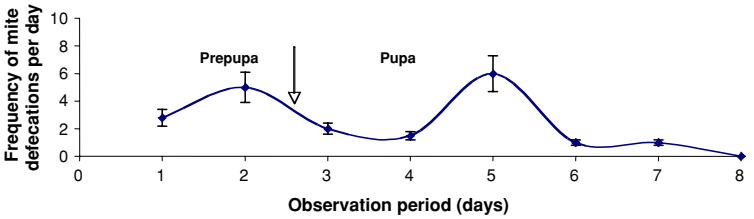

Fig. 2 Frequency of mite defecations in worker cells of Africanized bees (means ± SE). During the second and fifth day the mite defecate more frequently. The *arrow* indicates the beginning of the pupal stage

feeding activity of the mite and the defecation activity ($R = 0.75$). It becomes less and less active and spents more and more time upside-down on the feces. It descends onto the pupa exclusively to feed. After each feeding bout, the mite swiftly returns to the cell wall where it stops on the feces.

There is a high correlation between the position of the feeding site on the pupa and the position of the fecal accumulation on the cell wall ($R = 0.8$). The proximity of the fecal accumulation to the feeding site facilitates the feeding activity by the foundress mites and the progeny.

Displacement behavior of the bee larva

Longitudinal displacement was observed in 28.0% ($n = 25$) of the infested bee larva. Turning movements around the cell, from the bottom to the top, were clearly carried out by some larvae. The bee larvae showed the displacement behavior mainly on the second day (47.7 ± 22.5 min h^{-1}), with a total time of 874.9 ± 262.2 min day^{-1} ($n = 7$ individuals).

The displacement movement did not affect the growth of the bee larvae, because most of them completed development into the adult stage. However, the feeding activity of the mite during larva displacement was reduced (0.2 ± 0.2 bouts h^{-1}, $n = 7$ individuals) compared to the larva without displacement (0.3 ± 0.2 bouts h^{-1}, $n = 20$ individuals). Furthermore, damage to the mite was noticed in some of them. Mites found on the bottom of the cell were often injured, and such damage could be attributed to the displacement behavior of bee larva, that apparently moved toward the mite. Damage or injury marks on the dorsal shields of varroa were observed. In addition, in some cells with displacement larvae, the mite was found dead. In the uninfested cells (analyzed for bee brood development) no displacement movement was seen.

Movements of the larvae that occur when the prepupa moults into the pupa (without displacement) were observed. During the moult, bee movements to extend appendages, and deposit of the exuvium at the base of the cell, the mite was frequently pushed out of the fecal accumulation but quickly returned.

Discussion

Direct observations of varroa mite activity in worker brood cells of Africanized bees contribute to the understanding of the relationship between the host and the parasite. Using this method, we were able to describe a significant adaptation on the part of varroa mites in parasitizing the developing bee inside the capped cells.

Feeding activity

On most pupae only one wound was used as feeding site by haemolymph sucking. According to Donzé and Guerin (1994), the mother restricts her feeding to one site on the bee after pupation. This is remarkable in a number of aspects. The length of time invested by the mother on the feeding site suggests that opening of the wound on the pupa requires a high investment. According to Donzé et al. (1998), the mite invests a lot of energy in establishing the feeding site, as it will be the only one available in the cell. Since the female feeds regularly, a single feeding site located in the vicinity of the fecal accumulation is maintained. After feeding the mite returns to the fecal accumulation. This regulated behavior adopted by the mites in a cell, assures access to the feeding site when not in

use. It is of significance that varroa never feeds on the head of the pupa, thus avoiding possible damage to the developing stage (Donzé and Guerin 1994).

Position of the wound on the pupal stage

There are no records in the literature on the number of perforations made by varroa females as permanent feeding sites on capped Africanized bee brood. According to this method, the damaged cells surrounding the perforation of the mite for haemolymph meals become filled with trypan blue dye (Kanbar and Engels 2004a). In this study, most of the mites inflicted wounds on the abdomen of the pupa, specifically on the 2nd abdominal segment, mostly on the left side. According to Kanbar and Engels (2004b), on pupae many of the wounds are on the 2nd abdominal segment, indicating that the female mite has a preferred feeding site. According to Kanbar and Engels (2004b), in honey bee pupae the sternite of the 2nd abdominal segment is somewhat stretched over an underlying large haemocoelic cavity. On 16.3% of the pupae the wounds were located on the thorax. Kanbar and Engels (2004b) indicated that on 1/4 of the worker pupae the wounds were located on the mesothorax.

Construction of the fecal accumulation site

Accumulation of the mite feces appears as a white amorphous mass deposited on the cell wall. On the pupal stage, varroa shows a preference for defecation in the cell apex, usually close to the bee′s anal zone. Use of the posterior region of the cell by varroa and concentration of the feces at one site could reduce any odorous emissions, a factor which may be of significance in reducing detection by workers (Donzé and Guerin 1994). In addition, this site position on the cell is due to the fact that varroa shows negative geotaxis when defecating (Donzé and Guerin 1994). Once the fecal accumulation has been constructed, the mite becomes progressively rigid in its behavior routine. It only leaves the fecal accumulation to feed on the bee pupa, returning to it after each feeding bout usually to defecate. We found a high correlation between the position of the feeding site on the pupa and the position of the fecal accumulation site on the cell wall. All of this suggests that the fecal site serves as a great time and energy saver for the parasite in its race to maximize the number of fertilized offspring before bee emergence (Donzé and Guerin 1994). Fecal accumulations can also be used as a simple visual indicator to diagnose the presence of varroa (Erickson 1996).

Displacement behavior of the bee larva

At first sight the brood cell would appear to provide a good environment for mite reproduction (Donzé and Guerin 1994). However, the bee′s development limits the space available for the female mite and her offspring. A major change in space available for varroa inside the capped cell occurs when the prepupa moults into pupa. During bee movement, lasting some 30 min, to extend appendages and deposit the exuvium at the base of the cell, varroa is frequently pushed off the fecal accumulation (Donzé and Guerin 1994). Varroa is therefore not only confronted with the challenge of reproducing within the limited time span of bee development within the brood cell, but also with changes to the available space due to bee metamorphosis, a factor which increases the risk of mite mortality (foundress or offspring mortality) (Donzé and Guerin 1994).

In this study with Africanized bees, an additional movement was observed in the larval stage. Longitudinal displacements around the cell were carried out by 28.0% of the analyzed larvae. The feeding activity of the mites during these larval movements was reduced. Furthermore, damage to the mite was noticed in some of them. Such mite damage could be attributed to displacement of bee larvae that apparently moved toward the mite and pushed it against the cell wall.

The significance of the displacement behavior is not totally clear. Because bee larvae apparently pushed the mite against the cell wall, it could be considered as a defense mechanism of the larvae to varroa mites. There are no reports in the literature describing this type of displacement movement on capped brood. No displacement movement was observed in the uninfested cells. So far, hygienic and grooming behavior has been proposed as important resistant mechanisms of AHB to varroa. Hygienic behavior is related to bees opening up capped brood cells and removing the brood (Peng et al. 1987; Spivak 1996; Boecking and Spivak 1999). Grooming refers to the behavior in which the bees groom themselves and each other when they are irritated by the mites (Büchler 1994; Moretto and Mello 1999; Peng et al. 1987) and even kill them with their mandibles (Szabo et al. 1996). Both resistance mechanisms are described only for adult bees.

In conclusion, these results in worker brood of Africanized bees demonstrate significant adaptations on behalf of varroa mites in parasitizing the developing bee inside the capped brood cells. The varroa mother prepares a feeding site on the bee pupae and a fecal accumulation site on the cell wall. The fecal accumulation is situated near the feeding site on the pupa. The distance to the feeding site is minimal and mites could easily go up and down between the fecal accumulation and the feeding site. The feeding site on the pupa was found preferentially on the sternite of the 2nd abdominal segment and on the majority of pupae there was only one perforation present. In cells with displacement larvae, the feeding activity of the mites was reduced and damage to the mite was noticed in some of these cells. In a further study, we will focus on the mite's reproductive ability in AHB under these artificial conditions, and intend to compare it with mite reproduction in European bees.

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