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A comparison of the hygienic response of Africanized and European (*Apis mellifera carnica*) honey bees to *Varroa*-infested brood in tropical Brazil

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Abstract

In order to examine the significance of hygienic behavior for the tolerance to varroosis of Africanized honey bees, they were compared with non-tolerant Carniolans in tropical Brazil. Capped worker brood cells were artificially infested with living *Varroa* mites, and inspected some days later. Uncapping, disappearance of the introduced mite and removal of the pupa were recorded in a total of manipulated 3,096 cells during three summer seasons. The hygienic response varied between Africanized and Carniolan colonies, but this difference was significant only in one year, during which Africanized honey bees removed a significantly greater proportion of *Varroa* mites than European honey bees. A high proportion of the mites disappeared from artificially infested brood cells without damage to the pupae. The opening of the cell and the removal of the bee brood are independent traits of a graded response by adult workers towards mite-infested brood cells. We found a higher between-colony variation in the reaction towards *Varroa*-infested brood of Africanized honey bees compared to Carniolans. The overall similar response of the two bee types indicates that hygienic behavior is not a key factor in the tolerance to varroosis of Africanized bees in Brazil.

INTRODUCTION

The Africanized honey bee of Brazil (De Jong *et al.*, 1984; Moretto *et al.*, 1991; Rosenkranz, 1999) is one of the few examples of long-term tolerance to varroosis in *Apis mellifera* (Boecking and Ritter, 1993; Guzman-Novoa *et al.*, 1999; Rosenkranz, 1999). Although no treatment is carried out by Brazilian beekeepers, neither colony losses nor economic impact are recorded. The low rate of infestation in managed colonies has remained stable or even decreased during the past 20 years (Moretto *et al.*, 1991; De Jong 1996). The reason for this phenomenon, probably resulting from natural selection in the immense feral population from which beekeepers regularly trap swarms, is still unresolved. Of the various factors that have been proposed, only the high percentage of infertile mites observed in Africanized worker brood (Rosenkranz and Engels, 1994; Rosenkranz, 1999) can be regarded a key factor for this relatively balanced host-parasite relationship. Observations in the original host, *Apis cerana*, suggest that its tolerance to *Varroa* is in part due to mechanisms of behavioral defense like grooming and removal of infested brood (Rosenkranz *et al.*, 1993; Boecking and Spivak, 1999; Rath, 1999). Similar reactions have also been assumed for Africanized honey bees, reactions which have been considered less well expressed in non-*Varroa*-tolerant European honey bees (Guzman-Novoa *et al.*, 1999; Guerra *et al.*, 2000).

Hygienic behavior has been the topic of a number of recent studies on host defense in honey bees (Spivak, 1996; Spivak and Downey, 1998; Boecking and Spivak, 1999). Among the host behavioral factors which could influence the population dynamics of *Varroa* mites, the removal of infested brood would most clearly interrupt reproduction of the parasite. The heritability of the infested brood removal reaction is well documented (Rothenbuhler, 1964; Moretto *et al.*, 1993; Boecking and Drescher, 1998). Several assays have been described to quantify the hygienic response of a colony, among which the pin test and the freeze-killing method are widely used as an indication of the willingness of a colony to remove infested brood (Gramacho *et al.*, 1997, 1999; Boecking and Spivak, 1999). These developments have made hygienic behavior a favorite topic of programs to breed *Varroa*-tolerant strains of European honey bees (Boecking and Drescher, 1998; Spivak and Gilliam, 1998; Spivak and Reuter, 1998).

The suggested correlation between hygienic performance and reduction in parasite load needs to be proven in long-term studies. To quantify the removal response of honey bee colonies under defined conditions requires artificial infestation of single brood cells with female mites of defined origin. The testing of varroosis tolerant and susceptible honey bee strains should be done during the brood season in side-by-side experiments, using strong and normally breeding colonies. Because so far only a few experiments under such well-defined comparative conditions have been carried out with Africanized honey bees (Corrêa-Marques and De Jong, 1998; Guzman-Novoa *et al.*, 1999), we studied the hygienic response of colonies at a site in tropical Brazil across three summer seasons, using local Africanized honey bees and non-tolerant Carniolan colonies.

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MATERIAL AND METHODS

Study site, bees and mites

Experiments were performed during the summer seasons, December to February, of 1995 to 1998 in the apiary of the Department of Genetics, University of São Paulo in Ribeirão Preto, Brazil. We used eight colonies each of Africanized and Carniolan bees in Langstroth hives containing three to five brood combs. Mated queens of *Apis mellifera carnica* were imported from the University of Hohenheim, Germany. All the colonies were infested with *Varroa jacobsoni* mites of local Brazilian origin. These mites have not caused any colony losses amongst local Africanized honey bees during more than 20 years (De Jong, 1996). The Carniolans are non-tolerant to varroosis under temperate climatic conditions in Germany. Within eight weeks of introduction of the Carniolan bees to Brazil, we recorded infestation rates of the worker brood of 40-50%. Therefore, the Carniolan colonies suffered from high *Varroa* infestations and an insufficient adaptation to the tropical environmental conditions and had to be re-established every year using freshly imported queens.

Experimental design

For artificial infestation, caps of sealed worker brood cells about 24 h after cell sealing were opened at one edge using solvent-washed forceps. Female phoretic mites were carefully introduced into the cell on the tip of a sterilized needle. During the first year, mites of three different origins (same colony, Africanized and Carniolan colonies, respectively) were used to test the effect of foreign odor. The cells were resealed by using a drop of warm wax. Control cells were sham-manipulated without insertion of a mite. Twenty to 80 cells randomly selected in the center of a brood comb were treated in sequence. Immediately after handling, the combs were replaced into their original colonies. Three days later, and in some experiments only seven days later, the treated cells were inspected. Cells were marked and identified by use of a transparent plastic sheet. From three to five replicates were run per test series. A total of 1,362 artificially infested brood cells and 1,734 control cells were evaluated.

Test evaluation

After the scheduled time of incubation (three or seven days), we distinguished the following conditions of the manipulated brood cells: untouched, cell cap merely opened (‘cell cap opened‘), introduced mite removed but brood still present and resealed (‘only introduced mite removed‘), brood completely or partially removed (‘brood removed‘). Every seemingly untouched sealed brood cell was carefully examined to verify the presence of the introduced mite.

Data analysis

Differences between years and colonies in the proportion of cells to which bees responded were analyzed using a chi-square test of homogeneity. As the results revealed heterogeneity between years in the Africanized colonies, cross-table chi-square tests for every year were used to compare differences between bee types in their behavioral responses. The same statistic was used to analyze the effect of the origin of the mites and the hygienic reactions after three and seven days. All statistical tests were performed using the software modules of Statistica (StatSoft, 1994) and Microstat (Ecosoft, 1984).

RESULTS

Origin of the introduced mites

Hygienic response was independent of the origin of the *Varroa* mites ($P > 0.1$). Therefore, the data of mites of different origin were pooled.

Time course of hygienic reactions

There was no difference in the response to artificially infested brood cells in both Africanized and Carniolan colonies between the three- and seven-day inspections ($\chi^2 = 0.34$, $P = 0.56$ for Africanized bees; $\chi^2 = 0.25$, $P = 0.63$ for Carniolans). In the control cells (see below), higher percentages of brood were removed after seven days; however, these removal rates remained at levels below 5% (Figure 1).

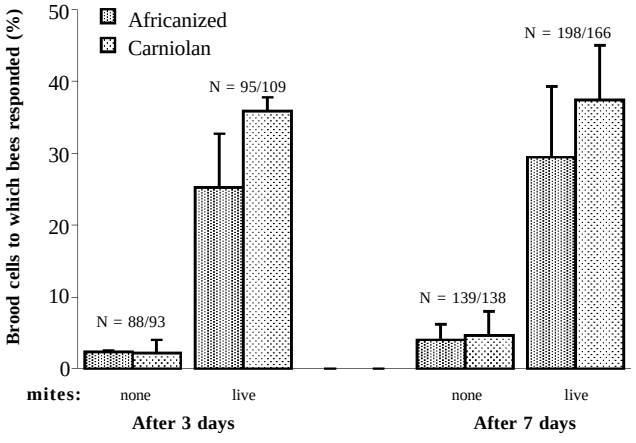

Figure 1 - Hygienic behavior of Africanized and Carniolan honey bees three and seven days after manipulation, respectively (N = number of manipulated brood cells). There was no significant increase in response to brood cells artificially infested with live mites between the 3rd and the 7th day ($\chi^2 = 0.34$, $P = 0.56$ for Africanized bees; $\chi^2 = 0.25$, $P = 0.63$ for Carniolans).

Treatment effects

The experimental manipulation of the capped brood cells (control: cell cap manipulated but no mite introduced) did in one case (Figure 2, 1996) release higher brood removal rates in Africanized honey bees than in European honey bees ($\chi^2 = 5.4$, P = 0.03). However, in all treatments, the percentage of brood cells to which bees responded was below 8%.

Comparison of Carniolan and Africanized colonies in different years

For this analysis all behavioral components (see below) were summarized. The year by year comparisons revealed significant differences in the hygienic response between bee types only for the first year ($\chi^2 = 5.2$, P = 0.03; Figure 2). In the following two years no statistical differences between bee types in their response to mites were detected ($\chi^2 = 0.92$ and 0.99, respectively; P = 0.34 and 0.32, respectively; Figure 2).

Different components of hygienic behavior

We analyzed the elements of hygienic behavior by recording 'cell cap opened', 'only introduced mite removed' and 'brood removed' (Figure 3). In very few cells was merely the cap manipulated or removed by the bees. The mite was missing and the cell had again been properly sealed by the bees in about one third of the artificially infested cells. In a minor fraction of cases, cells were found completely emptied, thus showing the full removal response. Both bee types performed the different traits of the hygienic behavior in similar proportions.

DISCUSSION

Importance of the experimental set up

We used artificial infestation of capped brood cells to evaluate the degree of *Varroa*-specific hygienic behavior in honey bee colonies. This method is widely used, even though the manipulation of the brood cells may elicit higher removal rates compared to naturally invaded brood cells (Boecking and Spivak, 1999). For artificial infestation, we only used brood cells containing an early prepupa to prevent the larva from spinning a cocoon around the introduced mite. The bees responded towards infested brood cells within a relatively short period. Maximal rates of manipulation were already recorded after three days. A longer exposure did not significantly increase hygienic response. This result confirms previous observations that bees usually react to *Varroa*-infested brood cells within a few days following infestation (Bär and Rosenkranz, 1992; Boecking *et al.*, 1992; Moretto *et al.*, 1993; Guerra *et al.*, 2000). Others (Vandame, 1996; Corrêa-Marques and De Jong, 1998; Boot *et al.*, 1999), however, described maximal rates of removal only after four to seven days. Possibly this disparity is due to secondary virus infections transmitted by the mite which only provide strong stimuli for removal reactions several days after infection of the larvae (Boecking and Spivak, 1999).

Only moderate differences in hygienic behavior between varroosis-tolerant Africanized bees and non-tolerant Carniolans

Comparative data on hygienic behavior in honey bees of different strain or origin are rare (Boecking and Ritter, 1993; Moretto *et al.*, 1993; Guzman-Novoa *et al.*, 1999; Guerra *et al.*, 2000). Our study represents one of the first long-term surveys of tolerant and susceptible colonies which were kept side by side. In more than 1,300 artifi-

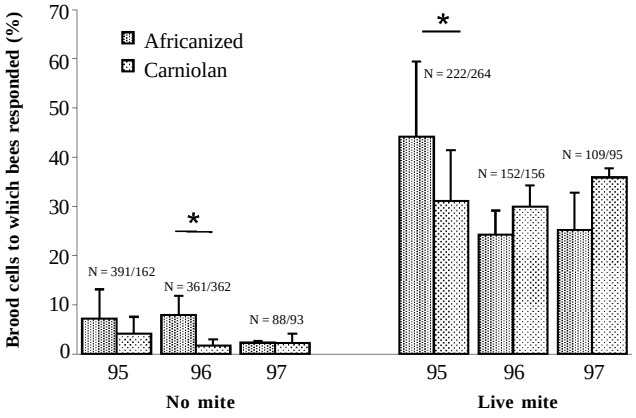

Figure 2 - Hygienic reactions of Africanized and Carniolan honey bees in different years (N = number of manipulated brood cells). Africanized honey bees responded to a significantly higher percentage of cells artificially infested with live mites only in 1995 ($\chi^2 = 5.2$, P = 0.03) and of control cells in 1996 ($\chi^2 = 5.4$, P = 0.03).

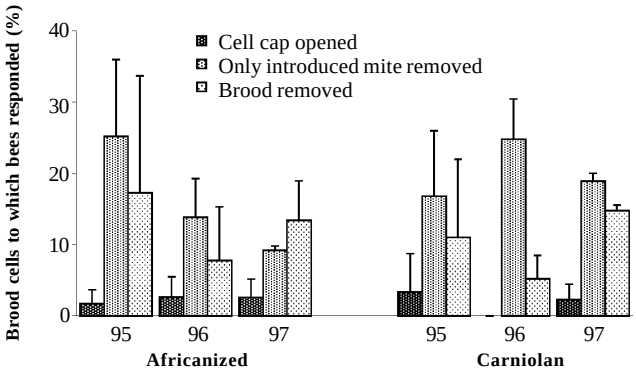

Figure 3 - Components of hygienic behavior in Africanized and Carniolan colonies (for summarized results see Figure 2). Mite removal is the predominant response to artificially mite-infested brood cells in both bee types.

cially manipulated brood cells studied during three years, we could detect significant differences in response of bee types only in one year. The hygienic response in the Carniolan colonies was uniform over the three-year period. In the Africanized colonies, there was higher between-year variation amongst colonies. Our data for Africanized bees are in the range of those of Guerra *et al.* (2000) at the same study site. However, our European Carniolan colonies had clearly more effective hygienic behavior compared to the *A. mellifera ligustica* bees used in that experiment.

Removal rates between 30 and 40%, as recorded in our experiments, may, in general, limit the growth of the *Varroa* population within a colony. But our susceptible Carniolan bees demonstrate impressively that this trait is not sufficient to prevent damage and collapse of the honey bee colony. From our data we cannot evaluate the contribution of hygienic behavior of Africanized bees to their well-known tolerance to *Varroa*. However, our data indicate that hygienic behavior is not the main factor for the differential varroosis tolerance of the Brazilian Africanized honey bee compared with *A. mellifera carnica*. Our results from tropical Brazil do not exclude that, at other locations, hygienic behavior may contribute more to the differential level of *Varroa* tolerance. Recent studies in Mexico (Vandame, 1996; Guzman-Novoa *et al.*, 1999) revealed a four-fold higher rate of removal of *Varroa*-infested cells by Africanized honey bees compared to European colonies. However, a comparison with our results is difficult because Vandame (1996) examined removal rates in naturally infested brood combs and used exuvial skins and feces of the mites as criteria for documenting former invasion by a *Varroa* female.

Different components of *Varroa*-specific hygienic behavior

In general, hygienic behavior is described as uncapping and removal of dead, diseased or parasitized brood (Boecking and Spivak, 1999). *Varroa*-specific hygienic reactions seem to be more complex and include repeated uncapping and resealing of infested brood cells (Bär and Rosenkranz, 1992; Rosenkranz *et al.*, 1993; Boecking and Spivak, 1999; Boot *et al.*, 1999). This may arise from the participation of several worker bees which may be specialized in different hygienic tasks (Boecking and Spivak, 1999).

The uncapping of brood cells represents the initial step in hygienic behavior (Corrêa-Marques and De Jong, 1998). Subsequently different options will lead to an interruption of the reproductive cycle of the parasite: the mite could escape from the uncapped brood cell, the mite could be removed by the bees without harming the pupa, and also the brood could be removed. In our experiments, we found a surprisingly high percentage of artificially infested brood cells with intact pupa but without a mite, and far fewer cells with removed brood. In total, the percentage of infested brood cells which were subject to hygienic behavior never exceeded 40% on average if all components of the behavior are included. No Africanized honey bee-specific enhanced performance in hygienic behavior could be detected.

Our results are within the range of responses published in previous studies (Bär and Rosenkranz, 1992); however, sometimes fewer mite-only removals and more removed pupae were recorded in these other studies (reviewed in Boecking and Spivak, 1999). The reasons for this discrepancy are unclear; probably, the brood removal rates of already-opened cells in other studies were dependent on secondary microbial infections. Cells containing a diseased pupa will not be resealed by the bees after taking out the mite. This peculiarity of mite removal without harming the healthy pupa that we recorded should be seriously considered in further research. For instance, by using freeze- or pin-killed brood, this mode of hygienic behavior is not seen.

Is hygienic behavior a promising candidate in selection for varroosis tolerance?

The mechanisms of *Varroa*-specific hygienic behavior and the effect of this trait within the multifactorial phenomenon of varroosis tolerance requires more research. In comparative studies, several crucial conditions have to be considered, as we have pointed out here. Present knowledge of the reasons for a balanced parasite host relationship between Africanized honey bees and *Varroa*, which may result from various features of both partners, suggests a highly complex system of interactions. To reveal more meaningful details is a promising challenge in studies of both the original host, *A. cerana*, and tolerant strains of the new host, *A. mellifera*. Rothenbuhler (1964) demonstrated that hygienic behavior resulted in American foulbrood tolerance. However, presently we have to conclude that this trait is not the major reason for the differential tolerance to varroosis of the Africanized honey bee in Brazil. Undoubtedly a certain percentage of *Varroa* females in the reproductive phase are eliminated by hygienic behavior. Taking into account the high percentage of infertile *Varroa* females in the worker brood of Africanized honey bees (Rosenkranz and Engels, 1994) only a small proportion of the original invaded mites will perform a successful reproduction. However, the lack of tolerance in our Carniolan bees is not because they do not perform hygienic behavior. The only slight difference in efficacy of this trait between tolerant Africanized and non-tolerant Carniolan colonies shows that other factors lacking in Carniolan bees must contribute to the pronounced varroosis tolerance of Africanized honey bees in the Neotropics.

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RESUMO

Com o intuito de examinar o significado do comportamento higiênico na tolerância à varroose de abelhas africanizadas, elas foram comparadas com as não tolerantes Cárnicas no Brasil tropical. Células de cria de operárias operculadas foram artificialmente infestadas com ácaros Varroa vivos e inspecionadas alguns dias depois. Desoperculação, desaparecimento dos ácaros introduzidos e remoção da pupa foram anotados em um total de 3096 células manipuladas durante três verões. A resposta higiênica variou entre as colônias africanizadas e de Cárnicas, mas esta diferença foi significante apenas em um ano, durante o qual as abelhas africanizadas removeram uma proporção significantemente maior de ácaros Varroa que as abelhas européias. Uma grande porporção de ácaros desapareceram das células das crias artificialmente infestadas, sem danos às pupas. A abertura da célula e remoção das crias das abelhas são características independentes de uma resposta graduada por parte de operárias adultas com relação a células de crias infestadas por ácaros. Nós encontramos uma variação maior entre colônias na reação de abelhas africanizadas à cria infestada por Varroa, quando comparadas a abelhas Cárnicas. A resposta geralmente similar dos dois tipos de abelhas indica que o comportamento higiênico não é um fator importante na tolerância à varroose de abelhas africanizadas no Brasil.

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