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Original article

**Bioassay for grooming effectiveness towards
Varroa destructor mites in Africanized
and Carniolan honey bees**

Pia AUMEIER*

Landesanstalt für Bienenkunde, University of Hohenheim, August-von-Hartmannstr. 13,
70599 Stuttgart, Germany

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Abstract – Grooming behavior is considered a varroosis tolerance factor of Africanized honey bees, but this behavior is difficult to evaluate directly within the honey bee colony. A laboratory bioassay was developed to measure the intensity and effectiveness of grooming responses by worker bees artificially infested with one *Varroa* mite. At a study site in tropical Brazil, the sequence of seven well-defined grooming reactions towards mites of different colonial origin was compared. In a total of 226 assays, Africanized bees responded significantly faster and more intensively than Carniolan workers. But there were no statistical differences in the removal of mites according to the bee types. Even extensive grooming behavior never resulted in damage or death of the mites. The possible use of the bioassay as a screening for the extent of the grooming behavior is discussed.

Africanized honey bee / behavioral trait / bioassay / grooming / *Varroa destructor* / Carniolan bee

1. INTRODUCTION

Grooming is a widespread strategy amongst vertebrates and arthropods to remove ectoparasites. Honey bees may perform allogrooming on a nestmate, for which a soliciting tremble-dance is shown, or

exhibit autogrooming in order to remove by themselves a parasite on their body surface. This behavior is regarded as an important trait in the defence against the parasitic mite *Varroa destructor* (Anderson, 2000; Anderson and Trueman, 2000)¹, as demonstrated in the original host species, *Apis cerana* Fabr.

* Correspondence and reprints

E-mail: bieneau@uni-hohenheim.de

Present address: Institute of Zoology, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

¹ The formerly described species *Varroa jacobsoni* encompasses several haplotypes which recently has been redefined as a complex of at least two different species: *Varroa jacobsoni* and *Varroa destructor*. The latter represents the species on which most *Varroa*-related work on *Apis mellifera* has been published.

(Büchler et al., 1992; Peng et al., 1987). High grooming effectiveness is considered to contribute also to varroosis tolerance in *Apis mellifera* L. (Boecking and Ritter, 1993; Boecking and Spivak, 1999; Božic and Valentincic, 1995; Guzmán-Novoa et al., 1999; Thakur et al., 1997), especially the Africanized honey bees of Brazil (Guzmán-Novoa et al., 1999; Moretto et al., 1993, 1995, 1997). This, however, was rarely compared at the same site with non-tolerant strains of European bees (Guzmán-Novoa et al., 1999; Moretto et al., 1993). Because grooming behavior was recommended for breeding programs to reduce the susceptibility of *A. mellifera* colonies to *V. destructor* infestation (Moretto et al., 1995), we carried out a comparative study in Brazil.

The grooming behavior of a bee infested with a mite is difficult to observe within a crowded hive. Direct records of grooming in observation hives are time-consuming to acquire (Moretto et al., 1993; Peng et al., 1987; Takhur et al., 1997), and the often used percentage of damaged mites collected in the debris only partly reflects the active grooming of bees towards living mites (Boecking and Spivak, 1999; Fries et al., 1996; Rosenkranz et al., 1997). Injured mites may also result from removal of infested brood cell content (Rosenkranz et al., 1997), wax moth caterpillars or ants (Guzmán-Novoa et al., 1999; Harbo and Harris, 1999). I developed a bioassay in order to quantify various components of grooming behavior in a short time interval and under constant ambient conditions.

2. MATERIALS AND METHODS

2.1. Bees and mites

All experiments were performed in 1997 in the University of São Paulo in Ribeirão Preto, Brazil. Eight Africanized colonies, originating from local swarms, and six colonies of *Apis mellifera carnica* Pollmann were used. The Carniolan colonies had been

established by introducing mated queens from Hohenheim (Germany) into Africanized colonies. They were only used after the worker progeny had replaced the initial bee population. In contrast to the Africanized honey bees, which in this region have survived without severe damage for more than 20 years (De Jong, 1996), the Carniolan colonies of the "Hohenheim source" proved to be highly susceptible to varroosis under temperate conditions. Even in Brazil with the predominant Japan/Thailand haplotype of *Varroa destructor* (Anderson, 2000; Anderson and Trueman, 2000) the mites showed high reproductive rates (Rosenkranz, 1999) and high infestation rates of the worker brood (40–50%) after 2–3 months.

Phoretic female mites were collected from five local Africanized colonies and five *Apis mellifera carnica* colonies, respectively. Since Carniolan colonies were obtained by requeening of Africanized colonies, the so-called "C-mites" (out of Carniolan colonies) formerly originated from the same mite population as the "A-mites" (out of Africanized colonies). All mites were randomly sampled from brood combs.

Mites were gently wiped off an anaesthetized bee with a needle and stored in a petri dish at 27 °C and high humidity, on damp filter paper, until used within an hour of collection.

2.2. Bioassays

Eight series of assays with Africanized, and six series with Carniolan honey bees were performed. Corresponding to the series, the bees were taken from eight Africanized and six Carniolan honey bee colonies. For each assay, three carbon dioxide anaesthetized worker bees (collected randomly from brood combs) were put into a glass petri dish and covered with perforated plastic foil (to prevent disturbance by air streams). After 30 minutes at 30 °C in an incubator in the dark, bees had recovered

from anaesthesia; they drank sugar water out of a plastic tip, fed each other or simply stood around. I only used these apparently normal bees in assays. If bees appeared still paralysed after 30 minutes or showed strong movements (running, fanning), the petri dish was rejected from the experiment. Spontaneous grooming behaviors without mite-contact were recorded in 10 assays with Africanized and Carniolan bees, respectively.

To start an assay, the plastic foil was lifted up just enough to apply very carefully one mite, sitting on the tip of a needle, onto the thorax of one of the three bees. In total, we performed 115 assays with Africanized honey bees (43 C-mites and 72 A-mites) and 111 assays with Carniolans (58 C-mites and 53 A-mites) under red light.

The behavior of all bees was recorded for three minutes.

2.3. Data recording

The duration and intensity of behavioral traits was obtained by distinguishing classes of behavioral components:

- “Weak cleaning” and “weak shaking” behavior consisted of lazily wiping antennae or briefly shaking the abdomen, lasting less than 5 seconds.

- “Intense cleaning” or “intense shaking” included more vigorous behavior, over a more than 5 second period. Intense shaking probably corresponded to the grooming dance, performed by parasitized bees in order to provoke allogrooming by nestmates (Peng et al., 1987; Thakur et al., 1997).

- Other types of intense response were “biting” of the mite,

- “rolling” by doing a somersault while hunting the mite and using all pairs of legs, or

- “attempting to fly”.

To evaluate the time course of grooming behavior, the number of grooming responses within and after the first 30 seconds were

registered. For this evaluation, only three assay series (41 single assays) with Africanized honey bees and four series (68 single assays) with Carniolans were performed.

The success of grooming reactions was evaluated by counting the number of mites which (i) were removed by the bee or actively left their host at least once, (ii) remounted their bee again, and (iii) finally gave up and, after 3 minutes, were found sitting on the petri dish. Each fallen mite was checked under a microscope at $\times 40$ magnification for damage. When a mite changed its host, the observation was discontinued and excluded from analysis, because I was only interested in the reaction of the focal bee to which the mite had originally been added.

2.4. Data analysis

Data is shown as means and standard deviations of the different series ($n = 8$ for Africanized bees and $n = 6$ for Carniolan bees, corresponding to eight and six colonies, respectively) in order to illustrate the range of activities and successes in mite removal. The influence of type of mites on the response of the Africanized and Carniolan bees, respectively, was tested by χ^2 assays. Relative frequency data for different behavioral traits was transformed via arcsine of the square root for comparisons of the two bee types by Student t -assays.

3. RESULTS

3.1. Spontaneous grooming behaviors

Uninfested Africanized honey bees and Carniolans never performed more than two “weak cleaning” reactions within 3 minutes.

3.2. Autogrooming versus allogrooming

Within the test time of 3 minutes, self-cleaning was the predominant response of

both bee types in the bioassay. 93% of Africanized bees and 87% of Carniolan bees expressed autogrooming. Only 4 out of 115 Africanized honey bees (3%) and none of the Carniolans were recorded as having solicited allogrooming. Allogrooming never dislodged a mite.

3.3. Colonial origin of the mites

Mites originating from the two bee types (A- and C-mites) did not elicit different grooming responses in Africanized or Carniolan honey bees ($p = 0.1$ for Africanized and $p = 0.3$ for Carniolans, χ^2). On average, Africanized bees performed 8.7 ± 8.3 grooming reactions against C-mites ($n = 43$) and 7.4 ± 6.8 against A-mites ($n = 72$). With Carniolan bees, we recorded 3.6 ± 2.7 reactions against C-mites ($n = 58$) and 3.3 ± 3.2 against A-mites ($n = 53$), respectively. Therefore, the data from A-mites and C-mites was pooled.

3.4. Frequency of grooming responses

On average, Africanized honey bees performed more grooming behaviors than Carniolans for all elements of grooming behavior (Fig. 1). In particular, reactions like “intense cleaning” and “intense shaking”, which in the laboratory assay primarily

led to removal of the mites, were more pronounced in Africanized honey bees than in Carniolans ($p = 0.02$ and 0.01 , respectively; t -test). Furthermore, Africanized honey bees repeated certain grooming reactions more often than Carniolans: up to 13% ($\pm 21\%$) of Africanized bees in contrast to only 2% ($\pm 3\%$) of the Carniolans repeated a certain behavioral component more than five times. Considering all behavioral components, the total number of responses was more than two times higher in Africanized bees compared to Carniolans (Fig. 1). The differences between the two bee types were significant ($p = 0.03$, t -test).

3.5. Time course of grooming behavior

Nearly 90% of all artificially parasitized Africanized bees, but only 66% of the Carniolans responded within 30 seconds after addition of a mite (Fig. 2: “continuous reactions” and “immediate reactions”; Fig. 3). Accordingly, a higher proportion of Carniolans performed delayed grooming only (after 30 s, Fig. 2). 63% of the promptly reacting Africanized bees performed more than two grooming reactions during these first 30 seconds compared to less than 25% in Carniolans (Fig. 3), while 20% more Africanized bees than Carniolans groomed themselves more than two times within 3 minutes.

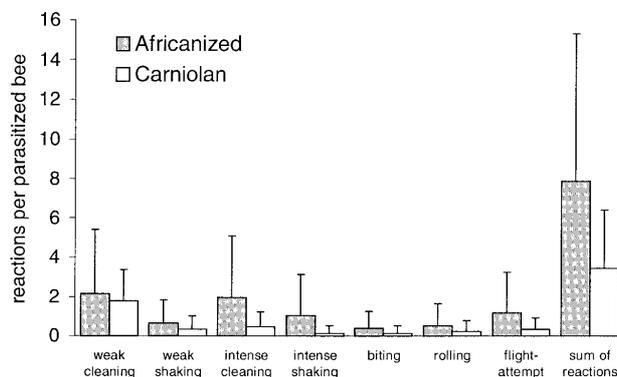
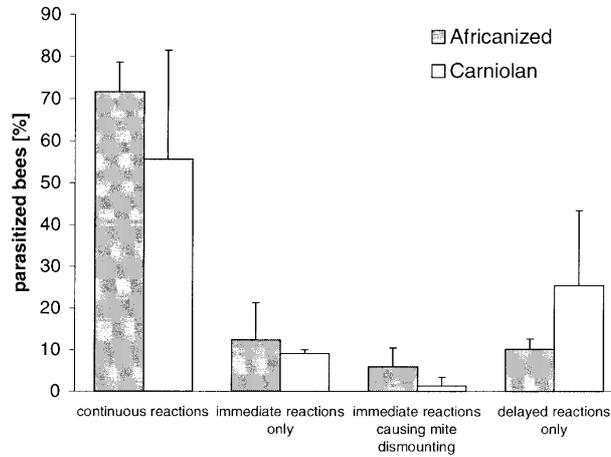


Figure 1. Mean number of various elements of autogrooming behavior. After addition of one mite, Africanized honey bees showed more grooming reactions than Carniolan bees (“sum of reactions”; $p = 0.03$, t -test), particularly in their intense reactions ($p = 0.02$ and 0.01 , for cleaning and shaking respectively, t -test, $n = 14$).

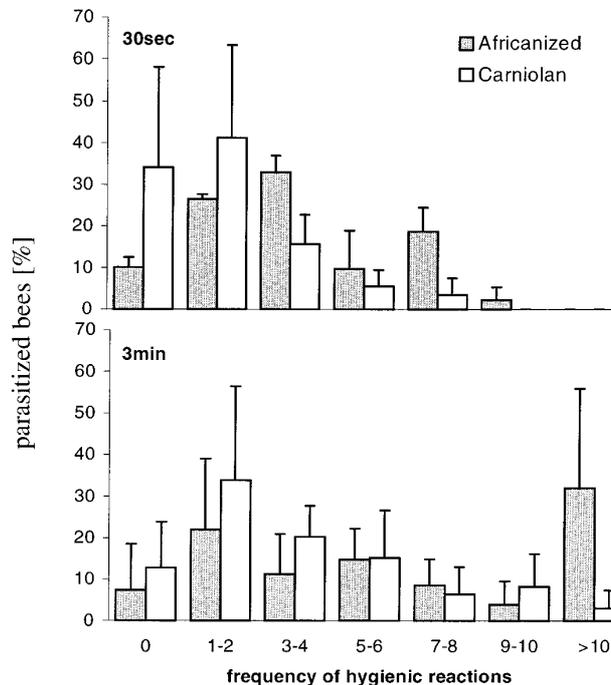
Figure 2. Start and persistence of grooming response after contact with one mite. There is a tendency for a larger number of Africanized honey bees to react either immediately (exclusively within 30 s after the beginning of the assay) or throughout the whole test period (“continuous reactions”). In contrast, more Carniolan bees only exhibited delayed reactions, commencing after 30 s.



Immediately reacting bees normally extended grooming over the whole assay period (“continuous reactions”, Fig. 2). In rare cases, grooming activities were limited to the first 30 seconds of an assay (“immediate reactions only”, “immediate reactions causing mite dismounting”). There were

also significant differences in the maximal number of reactions performed by the different bee types: more than 30% of Africanized bees, but only about 3% of Carniolans responded with more than 10 reactions during the 3 minute assay period (Fig. 3; $p < 0.01$, t -test).

Figure 3. Frequency of grooming reactions of Africanized and Carniolan honey bees within 30 seconds and within 3 minutes when exposed to one mite. Reactions are summed across all grooming behaviors. Within 3 minutes a higher percentage of Africanized honey bees responded more frequently ($p < 0.01$, t -test, $n = 7$).



3.6. Effectiveness of mite removal by grooming

Grooming by bees elicited intense movement of the attached mite. Africanized bees, on average, induced more mites to leave the host body than Carniolans (Fig. 4). However, in both bee types, about 80% of these once-removed mites remounted their bee again. Wiping off and remounting occurred repeatedly (up to five times) in both bee types. Finally, only 21 (out of 115) and 10 (out of 111) mites had no further contact with their Africanized and Carniolan hosts respectively once having been removed. This differences between the bee types in mite removal were not significant ($p = 0.08$; χ^2). No physical damage of these removed mites was visible, even if some bees had vigorously shaken and chewed the mites.

4. DISCUSSION

4.1. Assessment of the laboratory bioassay

Intensity and effectiveness of grooming behavior against *Varroa* mites is difficult to evaluate. Studies in full-sized colonies or

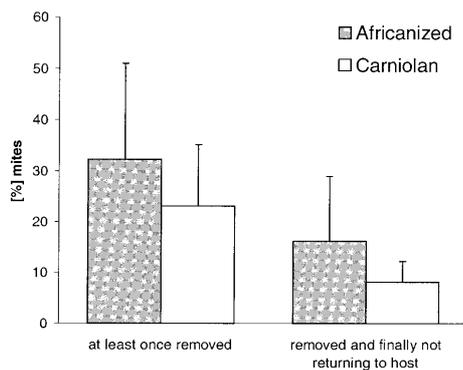


Figure 4. Success of grooming events: the majority of once dislodged mites returned to a bee. Finally, Africanized honey bees were as effective in removal as Carniolan ones ($p = 0.08$, χ^2 , $n = 14$).

observation hives (Božic and Valentincic, 1995; Fries et al., 1996; Moretto et al., 1993, 1997; Peng et al., 1987) are time-consuming to prepare and even then cannot guarantee continuous recording of one particular bee's behavior (Büchler et al., 1992; Peng et al., 1987; Thakur et al., 1997). On the other hand, reliability of indirect methods such as assessing the proportion of damaged mites in hive debris, is controversial (Boecking and Spivak, 1999; Corrêa-Marques and De Jong, 1996; Fries et al., 1996). Damage does not necessarily have to be attributed to grooming by adult bees, but may also originate from hygienic behavior in the brood or damage of already-dead mites within the sealed brood, on emerging bees, or by other organisms that are hive commensals or scavengers (Guzmán-Novoa et al., 1999; Harbo and Harris, 1999; Rosenkranz et al., 1997).

My laboratory bioassay enabled me to study various components of grooming response of individually mite-infested bees in a simple and economical way. In view of the data from observation hives in Mexico (Vandame et al., 1999), laboratory assay time was limited to 3 minutes. Vandame et al. (1999) mentioned that nearly all Africanized honey bees and more than half of European bees became agitated immediately after a mite had been placed onto them, with activity rapidly decreasing to nearly zero within 8 minutes. Moretto et al. (1993) also recorded about 80% of mite removal occurring within the first 5 minutes of half hour observations following mite addition to the bee. Probably, this temporal course of host response is caused by the mites, which rapidly choose places on the bee body where they do not stimulate grooming responses of their host (Boecking and Spivak, 1999). My assay system permitted exact quantification of a greater variety of behavioral patterns than described in recent literature (Božic and Valentincic, 1995; Ruttner and Hänel, 1992; Thakur et al., 1997), and it excluded external influences like number

of bees or brood, or changes of environmental conditions (literature cited in Boecking and Spivak, 1999). In contrast to observation hives, the bioassay facilitated recording of the activity and the condition of the infesting mite. Thus, I could not only observe the mite biting and crushing behavior of bees (Boecking and Spivak, 1999; Božic and Valentincic, 1995; Büchler et al., 1992), as described from infra-red video recordings by Thakur et al. (1997), but also easily check the consequences of biting for the mite afterwards.

Allogrooming could only be detected in rare cases in the artificial assay system of the bioassay, regarding only very small groups of bees. However, in contrast to autogrooming (Büchler et al., 1992; Fries et al., 1996), social grooming by nestmates seems to be of less importance in *A. mellifera*, where the rarely attracted allogroomers never attempted (Božic and Valentincic, 1995; Büchler et al., 1992) nor succeeded in catching or damaging a mite (Thakur et al., 1997; Vandame et al., 1999).

4.2. Africanized bees are better groomers

To a minor extent, my data is consistent with that of Vandame et al. (1999) and Moretto et al. (1993). The latter recorded Africanized honey bees to be on average seven times more effective in grooming than European honey bees (Moretto et al., 1993). In my laboratory bioassay, Africanized honey bees responded more vigorously to mite application than Carniolans. Differences were due to a faster reaction and a greater proportion of bees expressing a higher number of intense defence reactions in Africanized bees. Nearly all bees performed at least one component of grooming during the three minute assays. As assay bees had been randomly sampled from brood combs, we cannot answer whether grooming in general is a task restricted to a certain age or genetic line of specialists

(Kolmes, 1989; Thakur et al., 1997). However, in Africanized colonies, there are more persistent and intensive grooming bees.

Bees did not detect mites that were walking on the petri dish and away from a host. Even mites that appeared to be extremely disturbing to their hosts were instantly ignored when leaving the bee. Additionally, the responses of bees and mite removal rates in our assays were independent of the colonial origin of the mite. Obviously, the situation is different in *A. cerana*, in which the source of mites influences the response of bees (Büchler et al., 1992; Peng et al., 1987).

4.3. Does effective grooming of the bees influence the population dynamics of *Varroa mites*?

Direct observation of the test mite revealed that even vigorous host attacks neither mutilated nor killed any mite in the bioassay, confirming previous observations in *A. mellifera* races (Büchler et al., 1992; Moretto et al., 1993; Thakur et al., 1997). Besides, the grooming attacks could not deter mites from remounting a host. Overall, results from Mexico and Brazil (Vandame et al., 1999; this study) corroborate the view that even high grooming activity of bees has a remarkably low effect on the total number of mites expelled from the colony (11% and 8% after 8 minutes in Mexico for Africanized honey bees and European honey bees respectively, 16% and 8% after 3 minutes in Brazil for Africanized honey bees and Carniolans respectively). Thus, though bees are capable of injuring mites (Ruttner and Hänel, 1992), intensive grooming events do not necessarily mean injury to or effective elimination of mites (Büchler et al., 1992; Peng et al., 1987). This is confirmed by Corrêa-Marques and De Jong (1996), who detected very similar rates of mite damage for varroosis-tolerant Africanized and non-tolerant *A. mellifera ligustica* colonies. However, we have to take into account that, even if mites are not damaged visibly, they might

be considerably stressed by continuous grooming activities, which could also reduce their fitness (Fries et al., 1996).

My assay might be useful for simple and rapid screening of different strains of bees for grooming response to mite infection. However, findings of bioassays should be applied cautiously to full-size colony conditions. Therefore, it has to be proven in full-size colonies if the observed differences in grooming activity of the two bee types contribute to their different susceptibility towards varroosis.

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Résumé – Test biologique pour évaluer l’efficacité du comportement de toilettage vis-à-vis de *Varroa destructor* chez les abeilles africanisées et de race carno-lienne. D’après les observations faites sur l’hôte d’origine, *Apis cerana* Fabr., l’élimination du couvain infesté (comportement hygiénique) et le comportement de toilettage sont considérés comme des facteurs de tolérance à la varroose (Boecking et Spivak, 1999). Puisque le comportement de toilettage est difficile à quantifier directement dans la colonie, un test biologique de laboratoire a été mis au point pour évaluer l’intensité et l’efficacité des réactions de toilettage des ouvrières. A Ribeirão Preto, état de São Paulo (Brésil), nous avons comparé la réponse de toilettage d’abeilles africanisées tolérantes ($N = 115$) et d’abeilles carno-liennes, *A. mellifera carnica* Pollmann, non tolérantes ($N = 111$). Pour chaque test trois abeilles adultes ont été placées dans une boîte de petri en verre. Après une demi-heure d’adaptation, l’une des abeilles était infestée artificiellement avec un acarien

V. destructor phorétique provenant soit des colonies d’abeilles africanisées (acariens-A), soit des colonies de carno-liennes (acariens-C). Les diverses réactions comportementales ont été enregistrées durant trois minutes.

Les réponses des abeilles et le nombre d’acariens éliminés sont indépendants de l’origine de l’acarien ($p = 0,1$ pour les abeilles africanisées et $p = 0,3$ pour les abeilles carno-liennes, χ^2). Les abeilles africanisées ont répondu plus vigoureusement à l’application de l’acarien que les carno-liennes. Elles ont réagi plus vite (90 % contre 66 % des abeilles ont réagi dans les 30 premières secondes ; Figs. 2 et 3), ont accompli des réactions de défense deux fois plus nombreuses ($p = 0,03$, test- t , Fig. 1) et plus longues (33 % contre 3 % des abeilles ont accompli 10 réactions en 3 minutes, $p < 0,01$, test- t , Fig. 3). Les abeilles africanisées ont éliminé deux fois plus d’acariens que les carno-liennes (16 % contre 8 %, $p = 0,08$, χ^2 , Fig. 4), mais le comportement de toilettage n’a jamais abouti à des acariens mutilés ou tués.

Ce test biologique permet un criblage rapide de la capacité d’autotoilettage des colonies d’abeilles. Les résultats indiquent un seuil de réaction plus bas et une activité de toilettage plus intense chez les abeilles africanisées suite à l’infestation par l’acarien *V. destructor*. Néanmoins, il n’y a pas de différences significatives dans le taux d’élimination en fonction de l’origine des acariens. Il faut donc tester dans les colonies si les différences observées dans l’intensité du toilettage peuvent contribuer à une sensibilité différente des deux types d’abeilles à la varroose.

abeille africanisée / comportement de toilettage / test biologique / *Varroa destructor* / tolérance / abeille carno-lienne

Zusammenfassung – Labortest zur Untersuchung der Effizienz des Putzverhaltens gegen *Varroa destructor* Milben bei afrikanisierten und bei Carnicabienen

(*Apis mellifera*). Nach entsprechenden Beobachtungen beim Ursprungswirt *Apis cerana* Fabr. werden das Ausräumen befällener Brut (Hygieneverhalten) und das "grooming-Verhalten" als Varroatose-Toleranzfaktor diskutiert (Boecking and Spivak, 1999). Da "grooming" direkt im Bienenvolk schwer zu quantifizieren ist, wurde ein Labor-Biotest entwickelt, mit dem verglichen werden kann, wie intensiv und effektiv Arbeiterinnen gegenüber *Varroa*-Milben reagieren. An einem tropischen Standort in Brasilien wurden damit tolerante Afrikanisierte Honigbienen ($n = 115$) und anfällige europäische *A. mellifera carnica* Bienen ($n = 111$) verglichen. Für jeden Test wurden drei adulte Bienen in eine Glaspetrischale gesetzt. Nach 30 Minuten Beruhigungszeit wurde eine der Bienen mit einer phoretischen *V. destructor*-Milbe künstlich infiziert. Die Milben stammten entweder aus Afrikanisierten (A-Milben) oder aus *Carnica*-Völkern (C-Milben). Jeweils drei Minuten lang wurden dann die verschiedenen Reaktionen der Bienen erfasst.

Die Reaktionen der Bienen und der Anteil abgestiegener Milben wurde nicht durch die Herkunft der Milben (A- oder C-Milben) beeinflusst ($p = 0,1$ für Afrikanisierte Bienen und $p = 0,3$ für *Carnica*-Bienen, χ^2).

Auf Milbenkontakt antworteten die Afrikanisierten Bienen intensiver als die *Carnica*-Bienen. Sie reagierten schneller (90 % bzw. 66 % der Bienen innerhalb der ersten 30 Sekunden nach Versuchsbeginn; Abb. 2, 3) und zeigten insgesamt doppelt so viele ($p = 0,03$; t -test; Abb. 1) und ausdauerndere Abwehrreaktionen (33 % bzw. 3 % der Bienen mit mehr als 10 Reaktionen innerhalb von 3 Minuten; $p < 0,01$; t -Test; Abb. 3). Nach Versuchsende waren bei den Afrikanisierten Bienen doppelt so viele Milben abgestiegen (16 % bzw. 8 %; $p = 0,08$, χ^2 ; Abb. 4), jedoch war keine Milbe verletzt oder tot.

Der Biotest ermöglicht ein schnelles Screening der Fähigkeit zum Autogrooming verschiedener Bienenvölker. Afrikanisierte

Honigbienen zeigten bei einer *Varroa*-Parasitierung eine geringere Reizschwelle und länger anhaltende Abwehrreaktionen. Dies führte jedoch nicht zu signifikanten Unterschieden im Milbenabfall zwischen den untersuchten Bienen. Inwieweit intensiveres "grooming" zur unterschiedlichen Anfälligkeit gegenüber der Varroatose beiträgt, muss daher in weiteren Untersuchungen direkt im Bienenvolk beurteilt werden.

Afrikanisierte Honigbiene / Labortests / Putzverhalten / *Varroa destructor* / Varroatoleranz / Carnicabiene

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