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Original article

Differences in grooming behavior between susceptible and resistant honey bee colonies after 13 years of natural selection

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Abstract – The present study was conducted to quantify at the individual level the grooming behavior of bees from resistant colonies and susceptible colonies. Experienced and naive bees from resistant colonies were compared to experienced and naive bees from susceptible colonies at the age of 4, 7, 15 and 21 days. In a total of 480 assays, resistant bees successfully groomed off 10 times more mites placed than susceptible bees. Worker of different ages are involved but the lowest percentage of grooming was observed in 21-day-old bees. The experienced bees from resistant colonies bees that have evolved in a natural environment removed significantly more mites (69.2%) compared to naive bees (51.7%) who had no contact with other older bees.

***Apis mellifera* L / *Varroa destructor* / bioassay / grooming behavior / natural selection**

1. INTRODUCTION

The ectoparasite mite *Varroa destructor* (Anderson and Trueman 2000) causes major damages to beekeeping worldwide (Boecking and Genersch 2008; Rosenkranz et al. 2010). Without periodic treatment to control the mite, most of the honey bee colonies in temperate climates would collapse within a 2-to 3-year period (Rosenkranz et al. 2010). However, the application of synthetic miticides is problematic due to their collateral effects on bees (Gregorc and Bowen 2000). Besides, they may cause the development of resistant mites (Milani 1999). Moreover, natural substances such as organic acids and botanical essential oils are used but they are not consistently effective (Rosenkranz et al. 2010). Actually, several cases of natural resistance of bees to mites are reported around the world (Locke 2016). Five mechanisms could explain this natural resistance. These are (1) infertility of mites on bee brood (Locke et al. 2014), (2) unattractive brood to the mite (Nazzi and Le Conte 2016), (3) rapid brood development time (Calderón et al. 2010), (4) removal of mite-infested brood (Villa et al. 2017)

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and (5) grooming behavior (Guzman-Novoa et al. 2012; Invernizzi et al. 2016; Pritchard 2016).

In Algeria, *Varroa destructor* was reported for the first time in 1981 and local beekeepers treat their colonies either with chemical acaricides or with essential oils (Loucif-Ayad et al. 2008, 2010). In 2006, we set up an experimental apiary consisting of 175 colonies with a uniform mite infestation of "Tellian" bees presumably derived from (*Apis mellifera intermissa*). Routine operations were done monthly to control the presence of a queen and general condition of colonies. Except for adding supers to prevent natural swarming or for honey production when needed, no further operations were done on these colonies. These colonies were managed in standard 10 frame Langstroth hive and equipped with a screened bottom board in order to estimate mite populations. After a loss of 88 colonies in the first 5 years and 35 others 3 years later, the size of the apiary has finally reached 36 colonies in 2011. After that, the number of colonies has fluctuated very little. So we can say that the size of the apiary has stabilized. The remaining colonies showed no symptoms of diseases other than *Varroa destructor* infestation and exhibited normal development, these colonies that survived mite without any treatment can therefore be considered honeybee population naturally resistant to mites **(**Mohammedi 2016). Up to this date, the reasons for survival of the honey bee colonies remain unknown. The natural mite fall was examined to check for evidence of a possible mechanism that is contributing to colony tolerance. These colonies maintained lower mite populations year around and showed high proportions (15, 3%) of fallen injured mites (unpubl. data). Since there were no ants or wax moth larvae present on the floor insert, any mite damage other than regular dorsal dimples was due to the bees. Mites with one or two regular dorsal dimples were not considered as damaged (Davis 2009). These observations suggested that our colonies could have developed mechanisms to inhibit the growth of mite populations (Moosbeckhofer 1992; Arechavaleta-Velasco and Guzman-Novoa 2001; Stanimirovic et al. 2003). One of the natural mechanisms of resistance against *Varroa destructor* seems to be the grooming behavior of worker bees (Guzman-Novoa et al. 2012). This behavior allows bees to remove *V. destructor* from their bodies via self-grooming and nest–mate grooming performed by nestmate workers (Peng et al. 1987). Evaluating grooming behavior in bees of a given colony is usually done indirectly by considering the proportion of injured mites collected on the floor of the hives (Vandame et al. 2002; Guzman-Novoa et al. 2012).

As the grooming behavior of a bee infested with a mite is difficult to observe within acrowded hive and direct records of grooming in observation hives are time-consuming to acquire (Peng et al. 1987; Moretto et al. 1993; Thakur et al. 1997; Aumeier 2001), grooming behavior at the individual level was performed in a laboratory studies based on observing the behavior of the infested bee placed in a Petri dish (Aumeier 2001; Guzman-Novoa et al. 2012).

In general, grooming behavior seems to be highly variable. Marked differences in the proportion of bees that could dislodge the mite between several studies conducted by Aumeier (2001), Guzman-Novoa et al. (2012), and Invernizzi et al. (2016) could be due to methodological differences which greatly affect the ability of bees to dislodge the mite. For instance, when bees are randomly sampled from combs, it is not possible to answer whether grooming is a task restricted to a certain age (Aumeier 2001).

So far, hygienic behavior against *V. destructor* has been shown to be dependent on bee age (Panasiuk et al. 2010), as is also the case for acariosis (Pettis and Pankiw 1998). Regarding the grooming behavior, no study has been conducted to date to determine if this behavior is also related to the age of the bees.

The present study was conducted to (1) quantify at the individual level the grooming behavior of resistant bees by comparing them to susceptible bees, (2) to determine the age of worker bees involved in this grooming behavior, and (3) to show if the grooming behavior causes mite mutilations.

2. MATERIALS AND METHODS

The experiment was conducted on the campus of Boumerdes University, Algeria, from March to October 2017.

2.1. Origin of bees

2.1.1. *Resistant bees (RB)*

Four resistant colonies out of 36 were used as bee suppliers. These selected colonies showed high proportions of fallen injured mites.

2.1.2. *Susceptible bees (SB)*

Bees from 4 colonies heavily infested are used as a control. These colonies were treated against *V. destructor* with synthetic acaricide (Bayvarol©) during the autumn before experimentation. They were chosen for their good general condition and the size of the bee population.

2.2. Source of the mites

To harvest mites without disturbing colonies that are relatively aggressive, inserts made of 2 mm mesh screen wire, were placed under the brood nests to cover the whole bottom board of the hives. The edges of these inserts were coated with a very thin layer of Vaseline to prevent the dropping but still living mites from escaping. In order to collect enough mites, we used all the colonies at our disposal (4 resistant colonies and 4 susceptible colonies heavily infested). Active mites that had fallen onto the bottom-board traps were collected every 2 h. This short collecting interval of mites prevented an environmental influence and damage to mites due to decomposition. Mites were examined carefully under a stereomicroscope at 40x magnification. All types of injuries were considered, except regular dorsal dimples (Davis 2009). Collected mites were stored in a Petri dish at 27 °C and high humidity and used as soon as possible.

2.3. Experimental design

We selected frames containing enough capped brood that will emerge in one to 3 days (i.e., pupae with dark eyes and cuticle) to ensure that the required number of adults can be obtained. The combs were brought to the laboratory, placed in a screened cage and stored in an incubator at 33 °C, with a relative humidity between 70 and 80%. Emerging bees were pooled and immediately marked with different water-based Posca® pen on their upper thorax. The same procedure was done with susceptible bees. To distinguish them susceptible bees were marked with different water-based Posca® pen.

2.3.1. *Experienced bees from resistant colonies (ERB) and experienced bees from susceptible colonies (ESB)*

After being paint marked, approximately 600 resistant honey bees were distributed equitably among the 4 resistant colonies (150 individuals per colony). For the purpose of the experiment, a hundred marked bees were removed at 4, 7, 15 and 21 days. The same procedure has been applied for the susceptible bees. The "experienced bee" status has been assigned to these workers who evolved under normal conditions of a colony.

At the end of their stay in the colony resistant and susceptible bees are considered respectively as experienced bees from resistant colonies (ERB) and experienced bees from susceptible colonies (ESB).

2.3.2. *Naive bees from resistant colonies (NRB) and naive bees from susceptible colonies (NSB)*

Approximately 400 emerging resistant bees were equitably divided into four groups and kept in "classic" hoarding cages (12 × 10 × 4 cm) equipped with transparent and removable sides, ventilation holes, and provided with a vial of tap water, pollen and a piece of comb fastened in the upper part of cage. Each cage was equipped with two syringes of 2.5 ml adapted as feeders to provide water and sucrose solution (50% w/w). During the experiment, a batch of 4 resistant bee cages and 4 susceptible bee cages are maintained at controlled conditions (30 ± 1 °C, 50–70 % relative humidity, darkness) and all food was provided ad libitum. The bees will be used at 4, 7, 15 and 21 days. The "naive bee" status has been assigned to these workers who stayed in the cage, evolved under laboratory conditions and were in contact only with a single cohort of bees. At the end of their stay in the cage, these bees were considered naive bees from resistant colonies (NRB) and naive bees from susceptible colonies (NSB).

2.4. Petri dish test and observations

Grooming behavior was evaluated at the individual level in a laboratory using a modified version described by Aumeier (2001). Individual Petri dishes (9 cm diameter) were prepared in advance of the assays by lining their bottom with circular piece of white filter paper to provide contrast, thereby facilitating observation of bees and mites. Briefly, we placed three bees, inside individual transparent-plastic Petri dishes for at least 5 min to acclimate, after which we placed an adult female mite onto one bee's dorsal thorax with a paint brush. Active vital mites that were able to attach the host immediately were used for the experiment. We did not use the same mite more than once. A stopwatch was started immediately upon application of the mite, and the bees were observed for 3 min. In this experiment, only the auto-grooming was measured (in the rest of the text grooming means auto-grooming). Our study did not consider the classification done by Aumeier (2001). The test was discontinued in the event that a bee successfully removed the mite before 3 min had passed. A successful removal of the parasite was measured by the number of mites that fell from the number of infected bees in each group.

Each fallen mite was observed under a stereomicroscope for any damages. Mites were considered injured by the bees if they were missing legs or parts of legs. Each Petri dish containing the threesome of bees and mite was tested just once for each time of exposure and considered as a replicate. Several replicates have been realized (see Table I for more details).

2.5. Statistical analyses

All statistical analyses were performed with the R-Statistical Program (R Development Core Team, Auckland, New Zealand).GLM (general linear model) from "stat" R package was used to describe the relationship between the binary response for grooming and the effect of origin (resistant vs susceptible), status of bee (experienced vs naive) their combination (origin × status of bee) on that response.

Significant differences between levels of factors were tested using the Tukey post hoc test in "multicomp" R package. Statistical analyses were carried out using the R statistical environment version 3.50, a free software environment for statistical computing and graphics.

3. RESULTS

3.1. Effect of honey bee origin on grooming behavior

The proportion of mites removed by resistant bees (60.4%) is about 10 times higher than by susceptible bees (06.7%) (Figure 1, P < 0.001).161 mites out of 480 were removed, all of them were observed individually under the microscope. No mite showed any apparent injury.

3.2. Effect of honey bee origin and their status on grooming behavior

Regardless of ages, ERB that have stayed in a colony removed significantly more mites (69.2%) compared to NRB (51.7%) who had no contact with other older bees (P = 0.00589). This difference was no significant (P = 0.606) between NSB and ESB since the rate of grooming mite was respectively 7.5% and 5.8% (Figure 2).

3.3. Effect of the age of honey bees, their origin, and their status on grooming behavior response

Resistant bees showed variable rate of grooming behavior according to their age and status. At the age of 4 days, the percentage of removed mite was not statistically different between ERB (80%) and NRB (53.3%) (P = 0.1294). ERB that were 7 days removed (76.7%) more mites than NRB (36.7%). This difference was significant (P = 0.0121). When aged of 15 days, ERB and NRB removed respectively 80.0% and 76.7% of mites. This difference was not significant (P = 0.989). When the bees were 21 days old, the percentage of removed mite was the same (40%) with ERB and NRB (P = 1.00) (Figure 3).

Table I. Number of repetitions of each experiment according to origin, age and status of bees. A total of 480 tests were performed. Thirty repetitions were made for each category of age (4, 7 15 and 21 days), for each status (naive vs experienced) and for each origin (resistant vs susceptible) of bees. NRB: *Naive bees from resistant colonies* ; ERB: *Experienced bees from resistant colonies* ; NSB: *Naive bees from susceptible colonies;* ESB: *Experienced bees from susceptible colonies*

Age (days) Origin		4	7	15	21	Total number of repetitions
Resistant bees (RB)	NRB	30	30	30	30	120
	ERB	30	30	30	30	120
Susceptible bees (SB)	NSB	30	30	30	30	120
	ESB	30	30	30	30	120
Total number of repetitions		120	120	120	120	480

Comparison between resistant bees and susceptible bees showed that their status improves the rate of groomed mites. ERB removed significantly more mites than ESB when aged 4 days ($P < 0.001$), 7 days ($P < 0.001$), 15 days ($P < 0.001$) and 21 days ($P = 0.0313$). The same trend was observed with naive bees. Indeed, NRB removed significantly more mite than NSB at 4 days ($P = 0.00581$), 7 days ($P = 0.0420$), 15 days ($P < 0.001$), and 21 days ($P = 0.0555$) (Figure 3).

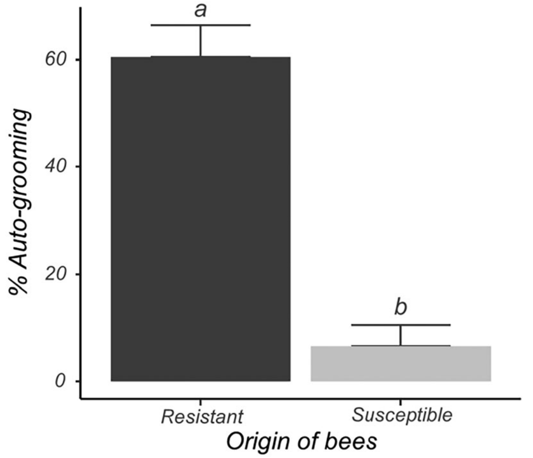

Figure 1. Percentage of worker bees (artificially infested with the parasite) manifesting grooming behavior according to their origin. Different letters indicate statistically significant differences (Tukey HSD test, $P < 0.05$, values represent % ± SEM). ($P < 0.05$ significant; $P < 0.01$ highly significant; $P < 0.0001$ very highly significant; $P > 0.05$ no significant difference).

4. DISCUSSION

Our study shows that in all cases, resistant bees successfully groomed off significantly more mites placed on their bodies (ten times more) than susceptible bees. Worker of different ages are involved but the lowest percentage of grooming was observed in 21-day-old bees. It is therefore related to the status (the task) of the worker. At 21 days, worker bees are supposed to be involved in outdoor tasks and are therefore less effective in grooming. Panasiuk et al. (2010) demonstrated that bees of different ages are involved in hygienic behavior but more frequently bees aged 6 to 10 and 16 to 21 days. Pettis and Pankiw (1998) studied the role of grooming behavior in bee *Apis mellifera* according to its age, they found that 90% of the grooming was observed when the bees are aged between 5 and 15 days.

Out of the 240 resistant bees observed, more than 60% dislodged the mite, which is much larger than found by Aumeier (2001), Guzman-Novoa et al. (2012) and Invernizzi et al. (2016). Aumeier (2001) found that 16% of Africanized bees and 8% of Carniolan bees could remove the mite. Guzman-Novoa et al. (2012) found that between 15.0 and 34.9% of the bees could dislodge the mite and Invernizzi et al. (2016) found that out of the 48 bees observed, only 6.3% could dislodge the mite. These marked differences between our results and those of Aumeier (2001), Guzman-Novoa et al. (2012) and Invernizzi et al. (2016) could be due to several factors like the *A. mellifera* strains and the

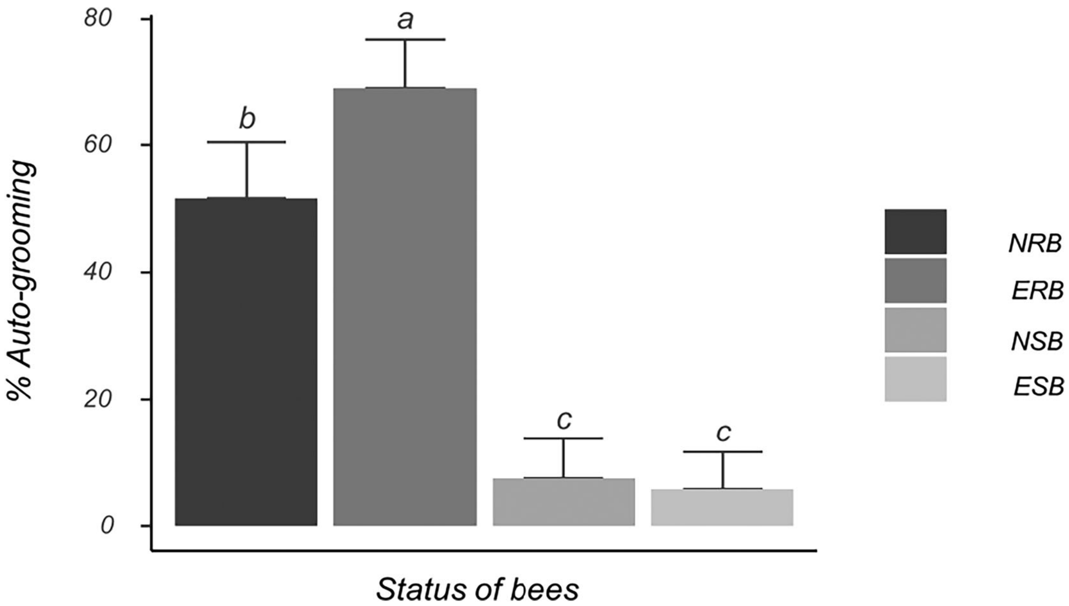

Figure 2. Percentage of worker bees (artificially infested with the parasite) manifesting grooming behavior according to their status. Different letters indicate statistically significant differences (Tukey HSD test, $P < 0.05$, values represent % ± SEM) (NRB: *Naive bees from resistant colonies*; ERB: *Experienced bees from resistant colonies*; NSB: *Naive bees from susceptible colonies;* ESB: *Experienced bees from susceptible colonies*). ($P < 0.05$ significant; $P < 0.01$ highly significant; $P < 0.0001$ very highly significant; $P > 0.05$ no significant difference).

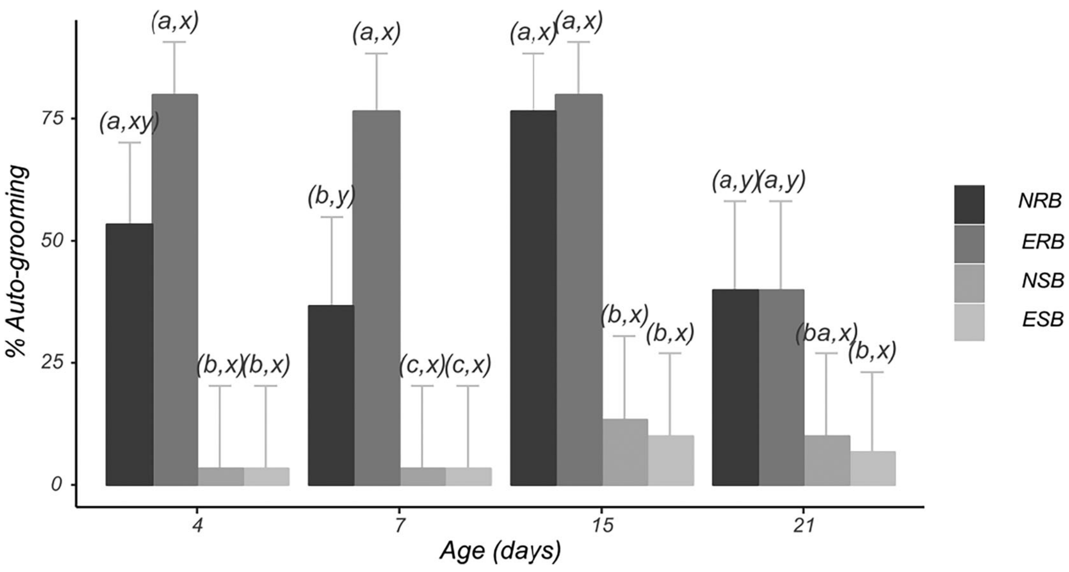

Figure 3. Percentage of worker bees (artificially infested with the parasite) manifesting grooming behavior according to their origin, age and status .The letters a, b ... concern the comparison of NRB, ERB, NSB, and ESB within the same age category; The letters x, y ... concern the comparison of NRB for 4 days to NRB at 7, 15 and 21 days (same for ERB, NSB and ESB). Different letters indicate statistically significant differences (Tukey HSD test, $P < 0.05$, values represent % ± SEM) (NRB: *Naive bees from resistant colonies*; ERB: *Experienced bees from resistant colonies*; NSB: *Naive bees from susceptible colonies;* ESB: *Experienced bees from susceptible colonies*). ($P <$ 0.05 significant; $P < 0.01$ highly significant; $P < 0.0001$ very highly significant; $P > 0.05$ no significant difference).

ability of mite to get out of reach of the legs of the bee during his attempts at grooming. Boecking and Ritter (1993) demonstrated that *V. destructor* prefers distinct attachment sites on *Apis mellifera intermissa* bee bodies to escape the attempts of grooming of worker bees. In our study, the resistant bee becomes very active just after the deposit of *V. destructor* on his body. This behavior has been observed also on Africanized bees (Vandame et al. 1999). During the first minute, we observed that all resistant bees insist to remove the mite from their bodies (auto-grooming) just after the deposition of mite. The removal of the mite is more difficult when it is hooked between the thorax and the abdomen because the bee cannot reach it. The location of the mite could therefore affect the effectiveness of the grooming activity (Bak and Wilde 2016**)**.

This test did not reveal a relationship between the injuries and the grooming behavior. It is interesting to note that no mite on the 480 tested was killed or injured. This observation agrees well with the results of several grooming behavior studies wherein very few damaged mites were reported (Buchler et al. 1992; Ruttner and Hänel 1992; Moretto et al. 1993; Rosenkranz et al. 1997; Thakur et al. 1997; Bienefeld et al. 1999; Aumeier 2001; Vandame et al. 2002; Wilde et al. 2003; Guzman-Novoa et al. 2012). However, it is not excluded that this grooming behavior is the cause of internal trauma that can eventually cause the death of mite after a few hours.

This eventuality could explain the large number of dead mites observed in the bottom board of the resistant stock colonies. To verify this hypothesis, it would have been interesting to compare the lifespan of the mite removed to those who escaped the legs of the bee.

Whatever their status, susceptible bees reacted in the same way to mite while the behavior of resistant bees is quite different depending to their status. Although the bees studied are from the same stock of bees, experienced bees from resistant colonies groomed significantly more mite (69.2%) than naive bees from resistant colonies (51.7%). In contrast to naive bees composed of a single cohort which have been kept in a totally artificial environment, experienced bees have evolved in their natural environment. So it seems that the environmental factor is the main explanation for this difference in the grooming behavior of naive and experienced bees. However in the case of our experiment, it is unclear how the environment affects bee grooming behavior. In social insects, the efficacy in performing tasks is shaped with experience and learning is involved in almost any task, including food type recognition and handling techniques (Chittka and Muller 2009). Therefore, it is possible that the bees in our research gained more experience in grooming behavior from contact with other parasitized bees in the hive. Moreover, interaction with other castes, the primer action of pheromones of the queen and brood as well, are factors that could play a role in the physiological maturation of the experienced bee which would have an impact on the grooming behavior. Although the difference in grooming behavior is clearly established between experienced and naive bees of resistant bees, it remains to prove the influence of the environment and how this influence is exerted on resistant bees and not on susceptible bees. In conclusion, since our results were obtained under controlled conditions, we should take precautions if we want to apply them to full-size colonies.

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AUTHORS' CONTRIBUTIONS

Contributions: AM designed the experiment and ND realized it; ND and AM interpreted the data and wrote the manuscript. MNM and OSZ performed statistical analysis. All authors approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Différences dans le comportement de toilettage entre les colonies d'abeilles sensibles et résistantes après 13 ans de sélection naturelle.

***Apis mellifera* L / *Varroa destructor* / essai biologique / comportement de toilettage / sélection naturelle.**

Unterschiede im Putzverhalten zwischen anfälligen und resistenten Honigbienenvölkern nach 13 Jahren natürlicher Selektion.

***Apis mellifera L* / *Varroa destructor* / Bioassay / Putzverhalten / natürliche Selektion.**

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