A direct assay to assess self-grooming behavior in honey bees (*Apis mellifera* L.)

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Received 15 June 2019 – Revised 5 March 2020 – Accepted 7 April 2020

Abstract – Grooming behavior confers resistance to honey bees against the mite *Varroa destructor* and thus is a mechanism of interest for studies of social immunity and breeding purposes. However, mites are not always available to conduct grooming assays and can introduce variability to the evaluations. Therefore, the objective of this study was to evaluate the use of wheat flour as an irritant in a laboratory assay to assess self-grooming behavior in honey bees. Presumed *Varroa*-susceptible and *Varroa*-resistant genotypes were used to determine the time it took the bees to initiate grooming instances and the proportion of bees that responded when exposed to either flour or a *Varroa* mite. Flour elicited significantly faster grooming responses and with a higher proportion of individuals in both genotypes in comparison with *V. destructor*. Also, significant differences between both genotypes were observed. It is concluded that flour can be used as an irritant instead of *V. destructor* to evaluate self-grooming behavior in bees in the laboratory.

honey bees / grooming behavior / *varroa destructor* / bioassay

1. INTRODUCTION

Varroa destructor is the most damaging parasite of honey bees. It has been found to be associated with high rates of colony mortality (Guzman-Novoa et al. 2010; Le Conte et al. 2010). Honey bees have developed defense mechanisms against parasitic mites like *V. destructor*, including grooming behavior, which consists in the use of legs and mandibles, as well as abdomen shaking to attempt to remove mites from their bodies (Peng et al. 1987).

Grooming behavior can restrain the growth of *V. destructor* populations in honey bee colonies (Arechavaleta-Velasco and Guzman-Novoa

2001). In fact, several studies have shown that certain bee genotypes such as the so-called Africanized bees (hybrids of *Apis mellifera scutellata*) are more resistant than European bees to *V. destructor*, in part due to differences in grooming behavior (Moretto et al. 1993; Guzman-Novoa et al. 1999; Aumeier 2001; Invernizzi et al. 2015). Clearly, grooming behavior is a mechanism of interest for studies of social immunity and breeding purposes. However, the assessment of grooming behavior is challenging. Indirect methods of assessing this behavior are based on counting the number of fallen and mutilated mites on sticky papers placed on the bottom boards of hives (Ruttner and Hänel 1992). However, these methods do not solely measure the effects of grooming behavior. Mites can fall and show body mutilations due to several other factors (Boecking and Spivak 1999). Thus, more reliable methods of measuring the behavior are needed for studies that require more precision.

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Manuscript editor: Yves Le Conte

Direct observation of grooming instances by bees can substantially reduce the unreliable nature of indirect methods. Direct methods of assessing the behavior include the visualization of bees grooming their bodies for a period of time when artificially infested with *Varroa* mites in observation hives (Peng et al. 1987; Fries et al. 1996) or in a Petri dish (Aumeier 2001). Molecular markers have been found to be associated with individual grooming behavior (Arechavaleta-Velasco et al. 2012). For example, *Neurexin* has been found to be overexpressed in bees that self-groom intensively (Hamiduzzaman et al. 2017), which validates the use of individual laboratory assays to assess self-grooming in bees. However, these methods depend on the availability of *V. destructor* mites at different times of the year. Moreover, *V. destructor* could also introduce variability in the evaluation of the behavior due to differences in their ability to get attached to the bee, as some could be damaged during the collection procedure as has been observed by the authors. Therefore, an assay to assess individual grooming behavior with an irritant that is not a *V. destructor* mite may be of aid to evaluate self-grooming behavior at different times of the year in a consistent manner.

The objective of this study was to evaluate the use of wheat flour as an irritant to assess self-grooming behavior in honey bees instead of using *V. destructor* mites. Presumed *Varroa*-susceptible and *Varroa*-resistant genotypes (Carniolan and Africanized bees, respectively) were used to determine the time it took the bees to initiate grooming instances and the proportion of bees that responded when exposed to either flour or a *V. destructor* mite.

2. MATERIALS AND METHODS

2.1. Source of honey bees and *V. destructor*

The worker bees used in this study were obtained from Africanized and European (Carniolan) bee colonies kept at the Honey Bee Breeding and Beekeeping Technology Centre of Mexico's Institute of Forestry, Agriculture and Livestock Research (INIFAP), in Villa Guerrero, Mexico (18°57'N, 99°38'W).

The source of Africanized bees included colonies previously used by Guzman-Novoa et al. (2012), which demonstrated significant increases in grooming behavior, mite removal success, and lower colony mite rates than colonies of European bee populations. The daily mite fall rates (on sticky papers) of the experimental colonies used differed significantly ($t_4=5.1$, $p < 0.01$) and were 45.7 ± 5.6 and 14.7 ± 2.4 mites for the European and Africanized bee colonies, respectively. The genotype of the colonies was confirmed by morphometric and mt DNA analyses of their bees (Nielsen et al. 1999). *V. destructor* mites were collected from heavily infested colonies as per Arechavaleta-Velasco and Guzman-Novoa (2001). Those colonies were different from the ones used to collect honey bees.

2.2. Self-grooming behavior trials

Adult honey bees were collected from the center of the brood chamber of the experimental colonies used in the self-grooming trials. Individual trials of grooming behavior were carried out in a laboratory at room temperature as described by Aumeier (2001) with modifications as per Guzman-Novoa et al. (2012). Briefly, each bee was gently placed inside a sterile Petri dish (100 mm × 15 mm, FisherScientific® Mississauga, ON, Canada) that was prepared in advance by lining its bottom with a circular piece of Whatman™ white filter paper (FisherScientific, Mississauga, ON, Canada) and covering it with plastic wrap, which was perforated 20–30 times with a nail (50 × 3 mm). Twenty milligram of wheat flour (Tres Estrellas®, Toluca, Mex, Mexico) per bee was measured in a scale (Denver Instrument®, Bohemia, NY, USA) before starting the self-grooming trials. Once the bee was introduced into the Petri dish, she was left for 2 min to become accustomed to the environment. Then, the plastic wrap cover was slightly lifted to place the wheat flour or one *V. destructor* mite on the thorax of each bee with a flat fine paintbrush (6 mm × 11 mm, DeSerres®, Oakville, ON, Canada). Each mite was used for one behavioral assay to avoid reusing mites that could have been injured by the self-grooming behavior of the bees,

thus affecting the results. For the control groups, Africanized or European bees were touched on the thorax with the bristles of a paintbrush of the same type as that used to apply the flour or the mites on the bees. The purpose of the control groups was to assess the effect of the touch by the paintbrush on the bees, which could result in grooming instances.

A stopwatch was started immediately after the application of the irritant, or the touch, and the bee was observed for 5 min. The time of first grooming response (movements of legs over the head, thorax or abdomen) was recorded. The proportion of bees that groomed at ≤ 60 s after exposure to an irritant or paintbrush touch was also determined. Bees of *Varroa*-susceptible and *Varroa*-resistant genotypes have consistently and significantly shown differences for these variables in previous studies (Aumeier 2001; Arechavala-Velasco et al. 2012; Guzman-Novoa et al. 2012). There was only one observer of grooming trials, which were conducted with 161 Africanized and 157 European bees.

2.3. Statistical analysis

(To compare treatments for time to react to the irritants or paintbrush touch by self-grooming, the data were subjected to a Kruskal-Wallis test and multiple pairwise comparisons using the Conover-Iman procedure (α of 0.05), as they did not comply with normality based on a Shapiro Wilk test. To compare treatments for proportions of bees that groomed at ≤ 60 s, contingency tables with chi-squared tests of independence (α of 0.05) were used, and adjusted residuals were calculated for post hoc analyses. The analyses were done with R version 3.4.3© (the R Foundation for Statistical Computing, 2017) and XLSTAT© (2019).

3. RESULTS

Significant differences were found between treatments for time to react to the irritants by self-grooming ($\chi^2 = 56.03$, $p < 0.0001$; Figure 1). Africanized bees reacted significantly faster than the control and European bees to flour (26.85 ± 2 s) and *V. destructor* (59.52 ± 7.3 s).

Figure 1. Mean time of first grooming response to an irritant ($\pm$ S.E.), a *V. destructor* mite (V) or 20 mg of wheat flour (F), or to a paint brush touch for the control groups (C) in bees of Africanized (AFR) and European (EUR) genotypes ($N = 318$). Different letters above bars indicate significant differences using a Kruskal-Wallis test and Conover-Iman procedure (α of 0.05). Non-transformed data are presented

European bees reacted to flour in 68.08 ± 10.56 s and to *V. destructor* in 118.77 ± 13.4 s. European bees started grooming themselves significantly faster than the control when exposed to flour, but not when exposed to *V. destructor*. Thus, flour elicited a significantly faster response, compared with *V. destructor* and the control in both Africanized and European bees ($\chi^2 = 28.31$, $p < 0.0001$; $\chi^2 = 12.63$, $p < 0.002$, respectively). No differences were found between the two genotype control treatments for time to start grooming ($p > 0.05$).

A significantly higher proportion of Africanized bees groomed at ≤ 60 s as a response to flour (0.94), compared with *V. destructor* (0.56) and the control (0.45) ($\chi^2 = 31.85$, $p < 0.0001$; Table I). Also, a significantly higher proportion of European bees groomed with flour (0.64) ($\chi^2 = 11.07$, $p = 0.004$), compared with the proportion of European bees that groomed with *V. destructor* (0.34) or the control (0.39). Lastly, a significantly higher proportion of Africanized bees responded to flour by self-grooming compared with European bees ($\chi^2 = 41.38$, $p < 0.0001$).

Table 1. Number and proportion of bees of Africanized and European genotype that groomed at ≤ 60 s after exposure to either a *V. destructor* mite, 20 mg of wheat flour, or a touch of a paintbrush (as control)

Treatment	N	Number bees that groomed at ≤ 60 s	Proportion of groomers
Africanized control	55	25	0.45
Africanized + flour	54	51	0.94 *
Africanized + <i>V. destructor</i>	52	29	0.56
European control	51	20	0.39
European + flour	53	34	0.64 *
European + <i>V. destructor</i>	53	18	0.34

Asterisks indicate a significant increase in the proportion of bees that self-groomed based on chi-squared tests and adjusted residuals ($p = 0.0083$) compared with the respective controls

4. DISCUSSION

The individual self-grooming assay described by Aumeier (2001) has been proven effective to identify differences in grooming behavior of different honey bee genotypes by evaluating various components of the behavior, including time of first response and the proportion of bees responding after being exposed to a *V. destructor* mite. The results of this study agree with those of Aumeier (2001) because Africanized bees groomed themselves faster and responded with a higher proportion of individuals to the irritation caused by *V. destructor* than European bees. But more importantly, when bees were exposed to flour, both genotypes initiated grooming instances significantly faster than bees exposed to *V. destructor* or the controls. Aumeier (2001) also reported differences for the intensity of self-grooming behavior between Africanized and Carniolan bees; thus, further studies on the differences in grooming intensity using flour as an irritant between tolerant and susceptible genotypes of bees to *V. destructor* parasitism would be of value to provide more information on the use of flour in individual grooming assays. Also, the effect of different quantities of flour or other irritants should be tested in future studies. Moreover, the potential impact of seasonal effects on self-grooming behavior should be taken into consideration to further investigate other variables influencing grooming behavior.

In addition to the above, a significantly higher proportion of both European and Africanized bees

reacted to flour at ≤ 60 s after its application compared with the control and the *V. destructor* treatments. Clearly, flour was the irritant that caused a faster response and with a higher number of individuals in both bee types, demonstrating that flour was able to provoke a self-grooming response consistently regardless of bee genotype. Additionally, significant differences between the two genotypes used were found for the two variables measured, which indicates that the use of flour can separate genotypes by level of grooming response. Moreover, no significant differences were found between the time of first response to *V. destructor* and the control in European bees, but a significantly faster reaction was observed with flour. Although flour could be a more effective irritant to assess self-grooming behavior, particularly in European bees, the results of this study should be taken cautiously, as the faster response with flour compared with *V. destructor* could overestimate grooming responses. Thus, the evaluation of more than one variable linked to grooming behavior, such as the proportion of bees that groomed, or the intensity of grooming, should be analyzed jointly with the time of reaction, to have a more comprehensive evaluation of grooming behavior. Also, to the best of our knowledge, there are no studies reporting on differences in the neural processes related to the perception of a *V. destructor* mite, pollen, or other particles on the bee's body that could elicit different grooming responses related to the type of irritant. The aim of this study was not to validate the use of the individual grooming assay with flour as the only

assay to evaluate resistance to *V. destructor* but to have an alternative to the self-grooming trial instead of using a *V. destructor* mite. Thus, the evaluation of *V. destructor* resistance through traits associated with grooming behavior in honey bees should be done using a comprehensive approach. Additional evaluations, such as mite population growth in colonies or proportion of mutilated mites (Morfin et al. 2020), could be used as a complement to the self-grooming assays.

Besides providing consistent results, another advantage of using flour as an irritant to evaluate grooming behavior in individual honey bees is that researchers would not be limited by the seasonal availability of viable *V. destructor* mites to perform the assay. Furthermore, flour could be used to evaluate grooming behavior associated to the resistance of honey bees to other parasites such as *Acarapis woodi*, as Danka and Villa (2003) noted that a higher proportion of bees that self-groomed were resistant to the tracheal mite. Also, the proposed assay could be used to assess the effect of stressors that could potentially affect self-grooming behavior, such as neurotoxins (Morfin et al. 2019). In conclusion, flour can be used as an irritant instead of *V. destructor* to evaluate self-grooming behavior in bees in the laboratory.

AUTHORS CONTRIBUTIONS

EG project planning and design; LE conducted field experiments; NM, EG, and LE analyzed the data; and NM and EG wrote the paper. All authors read and approved the final manuscript.

FUNDING INFORMATION

This study was funded by Mexico's Institute of Forestry, Agriculture and Livestock Research (INIFAP grant no. 22329) and the Technology Innovation and Research Program of the National University of Mexico (grant no. IN222203).

DATA AVAILABILITY

The dataset of the current study is available from the corresponding author on reasonable request.

COMPLIANCE WITH ETHICAL STANDARDS

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

Un test direct pour évaluer le comportement d'auto-toilettage des abeilles (*Apis mellifera* L.).

abeilles domestiques / comportement de toilettage / *Varroa destructor* / test biologique.

Ein direkter Test, um das Selbstgrooming-Verhalten bei Honigbienen (*Apis mellifera* L.) zu beurteilen.

Honigbienen / Grooming-Verhalten / *Varroa destructor* / Biotest.

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