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Article

Effect of hygienic behavior on resistance to chalkbrood disease (*Ascosphaera apis*) in Africanized bee colonies (*Apis mellifera*)

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Abstract:

The objective was to evaluate the hygienic behavior (HB) of Africanized honeybees (*Apis mellifera*) and its impact on resistance to ascospherosis caused by *Ascosphaera apis*. The HB and the population of adult bees and brood of 50 colonies were evaluated. In addition, colonies with high (>95 %) and low (<50 %) HB were inoculated with *A. apis* and in them, the number of broods with signs of ascospherosis (mummies) was determined for 17 days, data that correlated with their degree of HB. The susceptibility to *A. apis* of larvae from colonies with high and low HB in a common environment was also evaluated to separate environmental from genotypic effects. The degree of HB between colonies varied significantly (CV>36 %), with 20 % of the colonies showing high HB (≥95 %) and this did not correlate with the population of adult bees and brood. Colonies with high HB had significantly fewer mummies than colonies with low HB and there was a negative correlation between HB and number of mummies (r= -0.63, *P*= 0.02). In addition, larvae from colonies

with high or low HB were equally susceptible to the fungus. These results suggest that HB and larval susceptibility are not associated and that the main protection mechanism against *A. apis* in Africanized bee populations is HB. Therefore, the selection of colonies with high hygienic behavior could contribute to improving the health and productivity of honeybees.

Key words: *Apis mellifera*, *Ascosphaera apis*, Hygienic behavior, Larval susceptibility, Africanized bees, Yucatán.

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Introduction

Ascospherosis or chalkbrood, is a disease caused by the fungus *Ascosphaera apis*, which when it reproduces and sporulates in the larvae of honeybees (*Apis mellifera*) causes their mummification (black and white mummies), reduces the population size of their colonies, and in some regions can cause high losses in honey production[1,2].

In the presence of *A. apis* and other health problems of the brood, honeybees can respond with behavioral and physiological resistance mechanisms[3,4]. An important behavioral mechanism is hygienic behavior (HB), which consists of the ability of workers to detect, uncap and remove from inside the cells the brood that is sick or dead[5,6]. The evaluation of the HB level of a bee colony is based on sacrificing broods inside capped cells by puncture[7] or freezing[8] and determining the percentage of removal in a short period of time by the workers. It has been reported that colonies with very high HB (≥95 % removal of the dead brood in 24 to 48 h) show some degree of resistance to chalkbrood[9,10] and American foulbrood[11-13], and there is some evidence of resistance of highly hygienic colonies against the parasitic mite *Varroa destructor*[14] and the deformed wing virus[15]. It has also been argued that physiological mechanisms associated with the cellular and humoral immunity of bees could give them resistance against ascospherosis[4]. Therefore, it is not clear what is the contribution and relative importance of HB to the resistance of bees against chalkbrood, particularly in Africanized bee populations.

The HB has been reported to provide protection against the fungus *A. apis* in colonies of Africanized bees crossed with Europeans from South America[10]. However, it is unknown whether this also happens in Africanized bee populations in other countries. In addition, the

relationship between HB expression and brood susceptibility in Africanized bees infected with the fungus *A. apis* has not been studied. Generating such information would be very useful to design control strategies against the disease, such as establishing selective breeding programs.

In Mexico in particular, it is known that in certain regions the prevalence of chalkbrood can exceed 50 %, especially in humid areas[16], and that this disease can interact with others and cause bee colonies to collapse[17]. In addition, in Mexico, the relationships of HB with ascospherosis and the susceptibility of Africanized bee larvae to the fungus have not been studied.

Therefore, the objective of this study was to evaluate the degree of HB of Africanized bee colonies and its impact on the resistance and susceptibility of these insects to chalkbrood.

Material and methods

Place of study

This study was carried out in experimental apiaries of the Department of Beekeeping of the Campus of Biological and Agricultural Sciences of the Autonomous University of Yucatán, in Xmatkuil, Mérida, Yucatán, Mexico (20° 52' 3.00" N, 89° 37' 29.15" W). This region has a hot-subhumid climate with rainfall in summer (Awo), with annual rainfall of 985 mm, annual temperature of 26.8 ºC and annual relative humidity of 78 %[18].

Hygienic behavior and bee population

The studies were carried out in 50 colonies of commercial bees to which a morphometric analysis was carried out in order to confirm that they were Africanized[19]. The colonies were housed in Langstroth-type hives, distributed in five apiaries, and had different population conditions and food reserves, but without clinical signs of diseases. Colony bee populations were determined for both the capped brood area and the number of adult bees. To determine the brood area of each colony, two operators estimated the percentage of the area on both sides of each comb occupied by capped brood. The percentage area of the brood areas was converted to cm^2, considering the area that has a Langstroth-type comb on both sides (1,760 cm^2). In addition, the number of combs covered with bees was recorded and multiplied by

the number of bees that occupy a Langstroth comb in the brood chamber on both sides (2,430 bees)[20]. The measurements were made in the afternoons (> 1700 h) when most of the bees were inside the hives and the same operators participated in all the measurements.

The HB of the colonies was evaluated twice. The two assessments were conducted during July when there are no major blooms in the area and were made with an interval of 14 days. To estimate the HB level of each colony, a comb with capped brood with 3 to 4-day old pupae, identified by the white body and purple eyes[21], the most appropriate stage to determine HB[22], was selected. A galvanized sheet cylinder (8 cm diameter x 10 cm height) was placed over a compact capped brood area and 300 ml of liquid N_2 was poured to sacrifice the pupae inside the cells by freezing. When the N_2 evaporated, the cylinder was removed, and the frozen areas were photographed, and the comb was reintroduced into the colony being evaluated. The combs with the sacrificed brood of the experimental colonies were inspected 48 h after the previous procedure and the frozen areas were photographed again to record the number of dead pupae that were removed, and thus be able to determine the percentage of removal of the dead brood. The evaluations were carried out 48 hours after freezing because it is enough time to limit the reproduction and spread of an infectious agent, which is less strict than at 24 h and allows identifying the expression of HB in colonies not selected for this characteristic and naturally mated. Colonies that uncapped and removed 95 % or more of the frozen brood in the two tests were classified as highly hygienic (high HB) while colonies that removed 50 % or less of the frozen brood were considered to have low HB[8,12]. Subsequently, 10 colonies that presented a high HB and 10 colonies that presented a low HB were selected in order to evaluate their relative resistance to ascospherosis.

Effect of hygienic behavior on ascospherosis

The 20 colonies with high and low HB selected from the previous experiment were relocated to an isolated apiary and their queens were marked with indelible ink on the thorax for their identification. The bee populations of both groups of colonies were homogenized based on the colony that had the least number of bees, brood area, honey and pollen reserves. Therefore, at the beginning of the experiment, the 20 colonies had approximately the same number of combs covered with adult bees (8), capped brood (3), open brood (2), honey (2) and pollen (1). Additionally, it was corroborated that no selected colony presented clinical signs of chalkbrood (mummies present in the combs, floor, or entrance).

To obtain the fungus *A. apis*, black mummies (sporulated fungus) were initially identified from colonies outside the experiment. This clinical sign is pathognomonic of ascospherosis so there is certainty of obtaining the fungus from these mummies. Additionally, the

sporocysts of the fungus were observed under a microscope in mummy samples[23]. To induce *A. apis* infection in brood of the experimental colonies, the protocol of Flores *et al*[24] was followed. Three mummies per milliliter of distilled water were macerated. Each of the 20 experimental colonies was inoculated with 6 ml of the macerate diluted in 120 ml of sucrose syrup (1:1), which was supplied to each colony by means of Boardman-type feeders. In addition, two combs containing young larvae (open brood) on at least 80 % of their area, as well as the bees present in those combs, were sprayed with 6 ml of the same macerate diluted in 14 ml of sucrose syrup (1:1), supplying 5 ml of the inoculum on each side of each comb. The colonies were reviewed to record the number of black and white mummies present in the comb cells on days 3, 5, 7, 9, 12 and 17, post-exposure[25].

Susceptibility of larvae from high and low HB colonies to ascospherosis

In order to assess whether the differences in the number of mummies in the combs between colonies with high and low HB from the previous experiment were due to any extent to differences in the susceptibility of their larvae to the fungus *A. apis*, larvae from five colonies of each type selected at random were used to inoculate them and allow their development in a common environment. From each colony, a comb section (7 x 7 cm) containing an average of 264 ± 3.4 larvae 3 to 4 days old was cut with a knife. Immediately afterwards, racks were assembled, each containing a section from a colony with high HB and another from a colony with low HB, these sections and five receiving colonies that had a low HB were inoculated with the fungus *A. apis* as described above. Each comb assembled in this way was placed in the center of the brood chamber of a receiving colony in order to give the larvae the same nest environment, and that the probability of being removed by the HB of the bees from the receiving colonies was similar for both types of larvae. Making larvae and bees cohabitate in the same brood nest has been used successfully in the past to separate environmental from genotypic effects in studies of various bee behaviors, including HB[26-29]. The number of chalkbrood mummies in the comb sections was recorded on d 5, 9 and 13, post-exposure to the fungus.

Statistical analysis

Measures of central tendency and dispersion were obtained for the data from HB evaluations and population conditions of the 50 colonies. Pearson's correlation tests were also performed between the data from the first and second test of HB, as well as between those from the HB and those from the bee population, brood areas, and the number of mummies recorded in the

combs. The percentage of HB, brood areas and bee population of colonies with high and low HB that were selected for testing the effect of HB on chalkbrood, as well as the proportion of larvae clinically affected by ascospherosis of the two types of colonies that were used in the susceptibility experiment, were analyzed with Student's t-tests. The number of mummies from colonies with high and low HB and the effect of time on this variable were analyzed by means of repeated measures variance and Newman-Keuls comparison of means tests. Prior to the analyses, the percentage values of HB and mummified broods (susceptibility test) were transformed to square root of the arcsine and the number of mummies to logarithm, to ensure a normal distribution of the data. Statistical analyses were performed in the SAS program[30].

Results

Hygienic behavior and bee population

Table 1 shows the degree of HB and population conditions of the 50 colonies, as well as the variation for these parameters. Clearly, there was a wide range and variability for the degree of HB between the colonies evaluated (CV >36 %). However, it is noteworthy that 20 % of them had a high HB (≥95 %), 30 % had a low HB (≤50 %) and 50 % had an intermediate level of HB (51-94 % of frozen brood removal). The adult bee population was also highly variable (CV >37 %), but not the amount of brood in the colonies (CV <18 %).

There was a positive and significant correlation between the HB level of the first and second evaluation (r= 0.60, *P*= 0.0001), so the pupae freezing test showed repeatability. On the other hand, no relationship was found between the HB level and the number of adult bees (r= -0.03, *P*= 0.84) or with the capped brood area of the colonies (r= 0.02, *P*= 0.87), so it is presumed that these factors did not significantly influence the degree of HB of the colonies.

Table 1: Mean and dispersion values of hygienic behaviour, estimated bee population and brood areas in two tests to 50 honeybee colonies

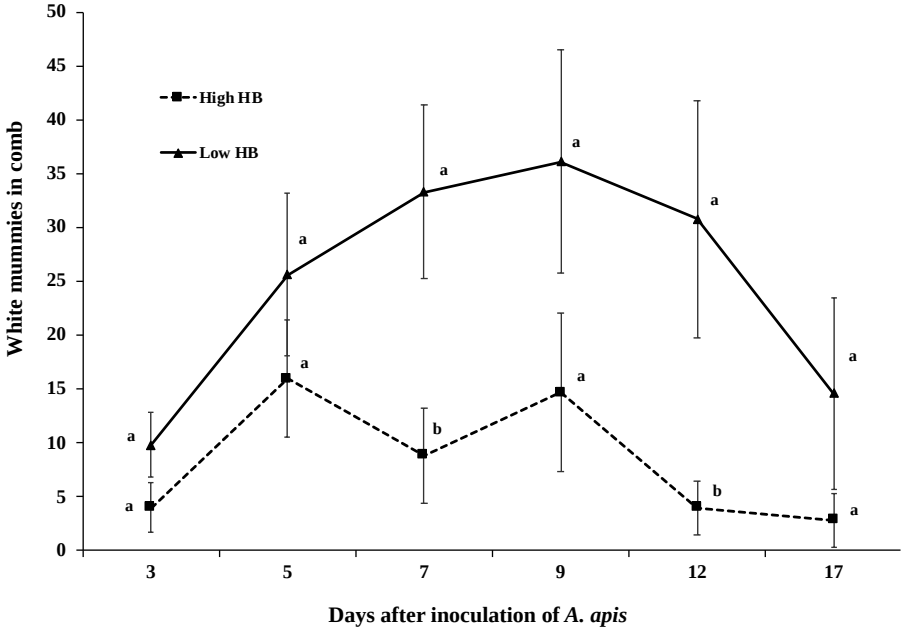
Descriptive statistics	Hygienic behavior (%)	Brood areas (cm^2)	Adult bee population
Mean	66.12	9,574.4	32,683
Standard error	0.48	33.8	245.43
Coefficient of variability, %	36.2	17.5	37.5
Minimum value	12.5	5,820	9,720
Maximum value	100	12,320	53,460
Range	87.5	6,500	43,740

Effect of hygienic behavior on ascospherosis

The colonies with high and low degree of HB that were selected for the test of relative resistance to chalkbrood differed significantly in their level of HB (t= 8.71, $P<0.0001$), but did not differ in terms of population of adult bees (t= 0.10, $P= 0.75$) or brood (t= 2.02, $P= 0.17$). Mean HB levels were 31 ± 0.81 and 97 ± 0.20 %, for colonies with low and high HB, respectively.

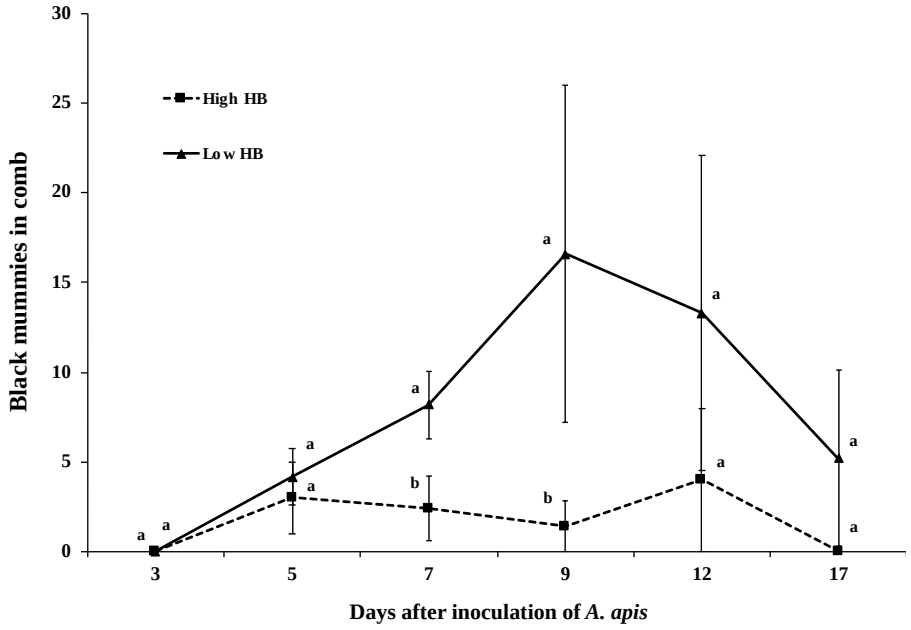
After being exposed to *A. apis*, the clinical manifestation of chalkbrood (presence of mummies in combs) was observed at 3 d post-exposure, and although the amount of affected brood was similar in both colony groups until day five post-exposure, colonies of high HB had significantly fewer white ($F_{1,76}= 32.1$, $P<0.0001$) and black mummies ($F_{1,76}= 10.8$, $P<0.001$) in the combs than the colonies of low HB. In addition, the number of black and white mummies in the combs of both colony groups decreased significantly and progressively between days 9 and 17 post-exposure ($F_{4,76}= 3.2$, $P= 0.01$ and $F_{4,76}= 2.6$, $P= 0.03$, respectively), but there was no interaction effect between the degree of HB and the days post-exposure to the fungus in white ($F_{4,76}= 0.7$, $P= 0.61$) and black mummies ($F_{4,76}= 0.95$, $P= 0.45$; Figures 1 and 2).

Figure 1: Number (mean ± SE) of white mummies recorded in the combs of colonies with high (■) and low (▲) hygienic behavior (HB) after inoculation with the fungus *A. Apis*



[ab] Different literals indicate significant differences ($P<0.05$) based on a repeated-measures analysis of variance and the Newman-Keuls comparison of means test, after transformation of the data to logarithm. Untransformed values are displayed.

Figure 2: Number (mean ± SE) of black mummies recorded in the combs of colonies with high (■) and low (▲) hygienic behavior (HB) after inoculation with the fungus *A. Apis*



[ab] Different literals indicate significant differences ($P<0.05$) based on a repeated-measures analysis of variance and the Newman-Keuls comparison of means test, after transformation of the data to logarithm. Untransformed values are displayed.

In addition to the above, a negative and significant correlation was found between the level of HB and the number of total mummies of the colonies inoculated with *A. apis* for the tests of resistance to chalkbrood (r= -0.63, P= 0.02). This result indicates that, the higher HB, the fewer mummies in the combs of the colonies, so this behavior seems to give resistance to honeybees against ascospherosis.

Susceptibility of larvae from colonies with high and low HB to ascospherosis

Regarding the susceptibility of larvae from colonies with high and low HB to the fungus *A. apis*, it was found that the proportion of mummies in the combs was not statistically different between both groups of larvae throughout the evaluation period (Table 2). In addition, there was no significant correlation between the HB level of the larval donor colonies and the percentage of mummies found in the combs post-exposure of the larvae with *A. apis* (r= 0.21, P= 0.55).

Table 2: Percentage (mean ± SE) of larvae from colonies with high and low hygienic behavior (HB) that clinically manifested ascospherosis post-exposure with *A. apis*

Post-exposure days with *A. apis*	High HB (n= 5)	Low HB (n= 5)	t	P
5	6.54 ± 0.77	5.32 ± 0.33	3.93	0.21
9	5.32 ± 3.24	4.14 ± 1.60	1.30	0.80
13	1.34 ± 1.81	1.46 ± 1.01	1.91	0.54

The t and P values were obtained from the analysis of data on the percentage of mummified broods transformed to the square root of the arcsine.

Discussion

Evaluations of the HB and strength of the population of the Africanized bee colonies studied showed wide variation as expected, but showed no correlation, which suggests that it is possible to identify bee colonies that vary in their HB regardless of their strength. These results coincide with what was reported in previous studies[10,12,31]. In addition, the N_2 test used to measure HB showed high correlation between repetitions, which indicates that it is reliable as previously demonstrated[32] and allows the categorization of colonies with different degrees of expression for this behavior. The frequency (20 %) of colonies that expressed a high degree of HB is within the ranges shown by European and Africanized bee colonies (10-31.5 %) in other regions[12,33-35]. And this low frequency could be increased by breeding queens from colonies with high HB, allowing their free fertilization, as Spivak and Reuter[12] and Bigio *et al*[31] have shown in practice. This is based on the fact that part of the variability of HB is of genetic origin[36,37], that HB is a highly heritable behavior[38-41] and that this is inherited through the mother[28].

Colonies selected for their high HB had significantly fewer broods with clinical signs of ascospherosis in the combs than colonies selected for their low HB. This was presumably because they removed more diseased brood from the combs than the colonies with low HB, and they did so progressively as the evaluation time passed. These results coincide with those of studies carried out in colonies with bees of European origin[42,43] and with those of a study carried out in colonies of Africanized bees crossed with Europeans[10]. The detection of larvae infected by *A. apis* depends on the perception of volatile compounds by bees[44-46], with variation in the detection thresholds of these compounds in workers. Bees of strains selected for high HB are more sensitive and react to the smell of these compounds more frequently than bees not selected for this behavior[47]. It has also been suggested that the efficiency of HB against chalkbrood depends on the early detection of larvae infected by the fungus prior to their mummification and sporulation, which limits the spread of the infection in the

colony[10]. Timely removal of diseased broods reduces the risk of sporulation and spread of the fungus[9]. This may explain why in colonies with high HB there were significantly fewer black and white mummies in combs compared to colonies with low HB. This conclusion is reinforced by the results of the correlation analysis that showed that, at a higher the degree of HB, the number of broods infected with *A. apis* in the combs of the bee colonies studied decreased significantly.

It has been speculated that the resistance of bees to brood diseases depends on the interaction of physiological resistance factors such as the immune response to different pathogens, the antimicrobial activity of the microbiota and larval foods, as well as HB and other defense mechanisms[48,49]. This study analyzed for the first time the susceptibility of larvae to *A. apis* in a common nest environment and found that larvae from colonies with high and low HB were equally susceptible to ascospherosis. Therefore, the results of the present study allow inferring that the level of HB expression and the susceptibility of larvae to *A. apis* are not associated, and support the hypothesis that although various factors and mechanisms could contribute to the resistance of bees against ascospherosis[50], HB is the most important of them. Therefore, it is suggested that efforts for the development of colonies resistant to brood diseases, such as ascospherosis, be focused on the selection of colonies with high HB.

The frequency of colonies with high HB from genetic improvement programs is higher than that found in unselected populations[12] and it has been reported that this behavior is associated with greater honey production[12,34,51] and greater resistance to diseases[9,12-15,36]; the latter is consistent with the results of the present study. Favorably, the selection and reproduction of queens from colonies with high HB seems to be sufficient to increase the frequency of colonies with high HB in bee populations[31,39,41], because HB is inherited mainly through the mother[28], so the implementation of selection and reproduction programs of queens whose colonies express a high HB would contribute to improving the health and productivity of the colonies.

Conclusions and implications

It is concluded that the degree of HB between colonies is variable, and that the population of bees and broods do not significantly influence the HB degree of the colonies. The frequency of colonies with high HB in the Africanized bee population analyzed would allow genotypes to be selected to increase the HB degree of bee populations in genetic improvement programs. Colonies with high HB had greater control of ascospherosis than colonies with low HB. The susceptibility of larvae to ascospherosis and HB do not appear to be associated factors. These results suggest that the main protective mechanism against *A. apis* in Africanized bee

populations is HB. Future research is needed to study the humoral and cellular immunity of bees against *A. apis*, as well as the mechanisms for its identification and associated factors, so that they are integrated into genetic selection and improvement programs in order to improve the health and production of honeybee populations.

Literature cited:

1. Gilliam M, Vandenberg JD. Fungi. In: Morse RA, Flottum K. Honey bee pest predators and diseases. 3ª ed. AI Root Co. Medina USA; 1997:82-99.

2. Zaghloul OA, Mourad AK, El MK, Nemat FM, Morsy ME. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Commun Agric Appl Biol 2005;70(4):703-714.

3. Evans JD, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, Hultmark D. Immune pathways and defense mechanisms in honey bees *Apis mellifera*. Insect Mol Biol 2006;15(5):645-656. https://doi.org/10.1111/j.1365-2583.2006.00682.x.

4. Larsen A, Reynaldi FJ, Guzmán-Novoa E. Bases del sistema inmune de la abeja melífera (*Apis mellifera*). Revisión. Rev Mex Cienc Pecu 2019;10(3):705-728. https://doi.org/10.22319/rmcp.v10i3.4785.

5. Boecking O, Drescher W. *Apis mellifera* removes *Varroa jacobsoni* and *Tropilaelaps clareae* from sealed brood cells in the topics. Am Bee J 1992;132(11):732-734.

6. Arathi HS, Ho G, Spivak M. Inefficient task partitioning among nonhygienic honeybees, *Apis mellifera* L., and implications for disease transmission. Anim Behav 2006;72:431-438. https://doi.org/10.1016/j.anbehav.2006.01.018.

7. Newton DC, Ostasiewski, NL. A simplified bioassay for behavioral resistance to American foulbrood in honey bees (*Apis mellifera* L.). Am Bee J 1986;126:278-281.

8. Spivak M, Downey DL. Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). J Econ Entomol 1998;91(1):64-70. https://doi.org/10.1093/jee/91.1.64.

9. Spivak M, Gilliam M. Hygienic behaviour of honey bees and its application for control of brood diseases and *Varroa*. Part II. Studies on hygienic behaviour since the Rothenbuhler era. Bee World 1998;79(4):169-186. https://doi.org/10.1080/0005772X.1998.11099408.

10. Invernizzi C, Rivas F, Bettucci L. Resistance to chalkbrood disease in *Apis mellifera L.* (Hymenoptera: Apidae) colonies with different hygienic behaviour. Neotrop Entomol 2011; 40(1):28-34. http://dx.doi.org/10.1590/S1519-566X2011000100004.

11. Danka RG, Villa JD. Preliminary observations on the susceptibility of Africanized honey bees to American foulbrood. J Apic Res 1994;33(4): 243-245. https://doi.org/10.1080/00218839.1994.11100878.

12. Spivak M, Reuter GS. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 1998;29:291-302.

13. Palacio MA, Figini EE, Ruffinengo SR, Rodriguez EM, Hoyo ML, Bedascarrasburne EL. Changes in population of *Apis mellifera L*. selected for the hygienic behavior and its relation to brood disease tolerance. Apidologie 2000;31:471-478. https://doi.org/10.1051/apido:2000139.

14. Ibrahim A, Spivak M. The relationship between hygienic behavior and suppression of mite reproduction as honey bee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor*. Apidologie 2006;37(1):31-40. https://doi.org/10.1051/apido:2005052.

15. Schöning C, Gisder S, Geiselhardt S, Kretschmann I, Bienefeld K, Hilker M, Genersch E. Evidence for damage-dependent hygienic behaviour towards *Varroa destructor*-parasitised brood in the western honey bee, *Apis mellifera.* J Exp Biol 2012;215(2):264-271. https://doi: 10.1242/jeb.062562.

16. Tapia-González J, Alcazar-Oceguera G, Macías-Macías J, Contreras-Escareño F, Tapia-Rivera J, Petukhova T, Guzmán-Novoa E. Ascosferosis en abejas melíferas y su relación con factores ambientales en Jalisco, México. Rev Mex Cienc Pecu 2020;11(2):468-478. https://doi.org/10.22319/rmcp.v11i2.4926.

17. Medina LM, Vicario-Mejía E. The presence of *Varroa jacobsoni* mite and *Ascosphaera apis* fungi in collapsing and normal honey bee (*Apis mellifera* L.) colonies in Yucatan, Mexico. Am Bee J 1999;139(10):794-796.

18. García E. Modificaciones al sistema de clasificación climática de Köppen, 5th. Ed. Universidad Nacional Autónoma de México (UNAM), México. 2004:90. http://www.publicaciones.igg.unam.mx/index.php/ig/catalog/view/83/82/251-1 Consultado: 25 Nov, 2020.

19. Nielsen DI, Ebert PR, Hunt GJ, Guzman-Novoa E, Kinee SA, Page RE. Identification of Africanized honey bees (Hymenoptera: Apidae) incorporating morphometrics and an improved PCR mitotyping procedure. Ann Entomol Soc Am 1999;92:167-174.

20. Delaplane KS, van der Steen J, Guzman-Novoa E. Standard methods for estimating strength parameters of *Apis mellifera* colonies. J Apic Res 2013;52(1):1-12. https://doi.10.3896/IBRA.1.52.1.03.

21. Jay CS. Colour changes in honeybee pupae. Bee World 1962;43(4):115-117.

22. Message D, Goncalves LS. Efeito das condições climáticas a da colônia no comportamento higiênico em abelhas *Apis mellifera* (africanizadas). Anais do 5o Congresso Brasileiro de Apicultura (Minas Gerais) 1980:55.

23. Guzmán-Novoa E, Zozaya-Rubio JA, Anguiano-Báez JR, Vázquez-Valencia I. Técnicas de diagnóstico de laboratorio de las enfermedades y parásitos de las abejas. En: Guzmán-Novoa E, Correa-Benítez A editores. Patología, diagnóstico y control de las principales enfermedades y plagas de las abejas melíferas. México: Editorial Yire; 2015:141-166.

24. Flores JM, Gutierrez I, Puerta F. A comparison of methods to experimentally induce chalk brood disease in honey bees. Span J Agric Res 2004;2(1):79-83.

25. Spivak M, Gilliam M. Facultative expression of hygienic behaviour of honey bees in relation to disease resistance. J Apic Res 1993:32(3/4):147-157.

26. Winston ML, Katz SJ. Longevity of cross-fostered honey bee workers (*Apis mellifera*) of European and Africanized races. Can J Zool 1981;59(8):1571-1575.

27. Guzman-Novoa E, Gary NE. Genotypic variability of components of foraging behavior in honey bees (Hymenoptera: Apidae). J Econ Entomol 1993;86(3):715-721.

28. Unger P, Guzman-Novoa E. Maternal effects on the hygienic behavior of Russian x Ontario hybrid honeybees (*Apis mellifera* L.*).* J Hered 2010;101(1):91-96. https://doi.org/10.1093/jhered/esp092.

29. Gashout HA, Guzman-Novoa E, Goodwin PH, Correa-Benítez A. Impact of sublethal exposure to synthetic and natural acaricides on honey bee (*Apis mellifera*) memory and expression of genes related to memory. J Insect Physiol 2020;121:104014. https://doi.org/10.1016/j.jinsphys.2020.104014.

30. SAS. SAS User´s Guide: Statistics (version 9 ed.). Cary NC, USA: SAS Inst. Inc. 2002.

31. Bigio G, Schurch R, Ratnieks FLW. Hygienic behaviour in honey bees (Hymenoptera: Apidae): effects of brood, food, and time of the year. J Econ Entomol 2013;106(6):2280-2285. http://dx.doi.org/10.1603/EC13076.

32. Espinosa-Montaño LG, Guzmán-Novoa E, Sánchez-Albarrán A, Montaldo HH, Correa-Benítez A. Estudio comparativo de tres pruebas para evaluar el comportamiento higiénico en colonias de abejas (*Apis mellifera* L.) Vet Méx 2008;39(1):39-54.

33. Oldroyd BP. Evaluation of Australian commercial honey bees for hygienic behaviour, a critical character for tolerance to chalkbrood. Aust J Exp Agric 1996;36:625-629.

34. Medina-Flores CA, Guzmán-Novoa E, Aréchiga FCF, Gutiérrez BH, Aguilera SJI. Producción de miel e infestación con *Varroa destructor* de abejas africanizadas (*Apis mellifera*) con alto y bajo comportamiento higiénico. Rev Mex Cienc Pecu 2014;5(2):157-170.

35. Gerdts J, Dewar RL, Finstrom MS, Edwards T, Angove M. Hygienic behaviour selection via freeze-killed honey bee brood not associated with chalkbrood resistance in eastern Australia. PloS one 2018;13(11):e0203969. https://doi.org/10.1371/journal.pone.0203969.

36. Arechavaleta-Velasco ME, Guzman-Novoa E. Relative effect of four characteristics that restrain the population growth of the mite *Varroa destructor* in honey bee (*Apis mellifera*) colonies. Apidologie 2001;32:157-174.

37. Lapidge KL, Oldroyd BP, Spivak M. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 2002;89(12):565-568. https://doi.org/10.1007/s00114-002-0371-6

38. Harbo JR, Harris JW. Heritability in honey bees (Hymenoptera: Apidae) of characteristics associated with resistance to *Varroa jacobsoni* (Mesostigmata:Varroidae). J Econ Ent 1999; 92(2):261-265. https://doi.org/10.1093/jee/92.2.261.

39. Boecking O, Bienefeld K, Drescher W. Heritability of the Varroa-specific hygienic behaviour in honey bees (Hymenoptera: Apidae). J An Breed Genet 2000;117(6):417–24. https://doi.org/10.1046/j.1439-0388.2000.00271.x.

40. Stanimirovic Z, Stevanovic J, Mirilovic M, Stojic V. Heritability of hygienic behaviour in grey honey bees (*Apis mellifera carnica*). Acta Vet (Beograd) 2008;58(5-6):593-601. https://doi.org/10.2298/AVB0806593S.

41. Pernal SF, Sewalem A, Melathopoulos AP. Breeding for hygienic behaviour in honeybees (*Apis mellifera*) using free-mated nucleus colonies. Apidologie 2012;43(4):403-16.

42. Gilliam M, Taber S, Richardson VG. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 1983;14(1):29-39.

43. Gilliam M, Taber S, Lorenz BJ, Prest DB. Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. J Invert Pathol 1988;52:314-325.

44. Swanson JAT, Torto SA, Kells KA, Mesce HH. Tumlinson JH, Spivak M. Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honey bee larvae. J Chem Ecol 2009;35:1108-1116. https://doi.org/10.1007/s10886-009-9683-8.

45. Zhao HX, Liang Q, Lee JH, Zhang XF, Huang WZ, Chen HS, Luo YX. Behavioral responses of *Apis mellifera* adult workers to odors from healthy brood and diseased brood. Sociobiol 2015;62(4):564-570.

46. McAfee A, Chapman A, Iovinella I, Gallagher-Kurtzke Y, Collins TF, Higo H, Foster LJ. A death pheromone, oleic acid, triggers hygienic behavior in honey bees (*Apis mellifera L.)*. Sci Rep 2018;8(1):5719. https://doi.org/10.1038/s41598-018-24054-2.

47. Masterman R, Ross R, Mesce K, Spivak M. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). J Comp Physiol A 2001;187(6):441-452. https://doi.org/10.1007/s003590100216

48. Guzman-Novoa E, Morfin N. Disease resistance in honey bees (*Apis mellifera L*.) at the colony and individual levels. In: Moo-Young M. editor. Comprehensive Biotechnology. 3rd ed. Amsterdam, The Netherlands: Elsevier BV; 2019;4:811-817. https://dx.doi.org/10.1016/B978-0-444-64046-8.00254-8.

49. Spivak M, Danka RG. Perspectives on hygienic behavior in *Apis mellifera* and other social insects. Apidologie 2020:1-16. https://doi.org/10.1007/s13592-020-00784-z.

50. Evison SE, Fazio G, Chappell P, Foley K, Jensen AB, Hughes WO. Innate expression of antimicrobial peptides does not explain genotypic diversity in resistance to fungal brood parasites in the honey bee. Apidologie 2016;47(2):206-215.

51. Wielewski P, de Toledo VAA, Nunes ME, Costa-Maia FM, Faquinello P, Lourenco DAL, *et al*. Relationship between hygienic behavior and *Varroa destructor* mites in colonies producing honey or royal jelly. Sociobiology 2012;59(1):251-274.