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VARIABILITY OF HYGIENIC BEHAVIOR IN BEE *APIS MELLIFERA MACEDONICA*

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Abstract

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The hygienic behavior is an essential factor for the selective improvement of resistant bees against the mite *Varroa destructor*. Bees with hygienic behavior remove the diseased or dead brood of closed cells. However, the expression of hygienic behavior varies greatly from month to month. In our work we examined the first year, for a period of four months, seven colonies of the Macedonian race (*Apis mellifera macedonica*) and the second year for a period of three months, 16 colonies of the same race. We found large variation in hygienic behavior each month in 17 of the 23 colonies (74%). Unlike in six of the 23 colonies (26%), in which was found a little variation of hygienic behavior (CV ≤ 15%), there was a positive correlation between the hygienic behavior and mite infestation on bee population. The hygienic behavior explains 32% of the variance of the mite infestation on bee population. Also, it was found that the hygienic behavior in the six colonies not affected by the strength of the colonies.

Key words: hygienic behavior, *Apis mellifera macedonica*, Varroa destructor, strength, variation

Introduction

The hygienic behavior is described as the uncapping and removal of dead, diseased or parasitized brood. Rothenbuhler (1964a) assumed a two-locus model for uncapping and removal of brood diseases and proposed that one locus controls the uncapping of brood cells containing dead pupae, while the second controls the removal of the cell contents. This model was re-evaluated by Moritz (1988) who suggested a more complex three- or more loci model for the removal behavior. Varroa-specific hygienic reactions seem to be even more complex and included repeated uncapping and resealing of infested brood cells (Rosenkranz et al., 1993b; Boot et al., 1999; Aumeier et al., 2000). This may be due to the participation of several genetically distinct worker bees which are specialized in different hygienic tasks. The removal of mite infested brood does not necessarily include the death of the mites, as it was observed that most mites seem to escape from the opened brood cells during the removal process (Boecking and Spivak, 1999). However, the removal of mites from the brood leads to an interruption of the reproductive cycle of the parasite, a prolonged phoretic phase or even the death of the mites (Rosenkranz et al., 2010).

The removal of mite-infested brood represents the main factor for the selective breeding of mite tolerant European honey bees (Spivak and Reuter, 1998a; Ibrahim and Spivak, 2006; Harris, 2007). Several methods have been described to quantify the hygienic behavior of a honey bee colony, of which the frozen brood test and the pin test are now widely used (Spivak, 1996a; Spivak, 1996b; Boecking and Spivak, 1999 and Gramacho et al., 1999). In both tests, a certain number of brood cells are killed either by freezing or by piercing; after a defined period the percentage of removed dead brood cells is recorded and used as a measure for hygienic behavior. However, several restrictions exist for successful selection of a Varroa-specific hygienic behavior (Rosernkranz et al., 2010). It is not clear whether the mechanisms for the detection of dead brood are the same as for the detection of mite-infested cells and whether there is sufficient correlation

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between “killed brood” and “removal of mite infested brood cells” (Boecking et al., 2000).

The expression of hygienic behavior is known to be strongly influenced by environmental factors. For example, weak colonies, or a lack of incoming nectar have been shown to reduce the removal response to mite-infested and dead brood cells, respectively (Momot and Rothenbühler, 1971; Boecking and Drescher, 1993; Spivak and Gilliam, 1993; Spivak, 1996b). Moreover, the results of Boecking and Drescher (1998) demonstrate that the rate of removal of mite infested or dead brood within a particular colony even under the same environmental conditions is not always consistent between assays (Hoffmann, 1996; Rodrigues et al., 1996; Spivak and Downey, 1998).

The aim of this study is to investigate the variations in the expression of hygienic behavior of *Apis mellifera macedonica*. Moreover, the hygienic behavior and the mite infestation were studied in two periods. We studied the colonies which showed a stable hygienic behavior and we compared this behavior in relation to infestation by the mite on bee population. Finally we compared the hygienic behavior in relation to the strength of the beehive.

Materials and Methods

The study was conducted in two periods. The first measurement period (May 2011 - August 2011) we used seven colonies out of twelve that survived the winter, without treatment against the mite. All colonies had sister queens and similar brood and bee population. The hygienic behavior and the percentage of mite infestation in brood and adult bees were calculated.

In the second period (May 2012 - July 2012) 16 new colonies were used with queen sisters, which survived the winter after chemical control of mite, of the 19 original colonies, were created in October of 2011. The new colonies were produced by rearing from the hive of the first period which had the highest percentage of hygienic behavior. The 16 colonies were treated with coumaphos to control the mite. After treatment, the infection rate of the mite was zero. In each hive there was calculated hygienic behavior, the brood, the population and the degree of mite infestation.

Also only the hygienic behavior, in October and November of 2012, in 10 additional colonies of the apiary was measured. These colonies were headed by naturally mated queens.

To estimate the mite infestation of the bee population 3 samples of 100 adult bees, collected from three frames of brood (a total of 300 bees) were placed into a vial containing 25% alcohol solution. After 24 hours the dead bees and mites were counted. To estimate the infestation level of brood, three samples of 200 sealed cells from three frames of brood were opened diagonally and the infestation level was found according to Pappas and Thrasyvoulou (1988).

The degree of hygienic behavior was performed by freezing (- 20°C) for 24 hours a section of 100 sealed brood cells and repositioned in the original hive (Taber, 1982; Taber and Gilliam, 1987; Spivak and Downey, 1998). In the frame of the sealed brood there was a cut with a utility knife 120 cells (6 cells x 20 cells) in the shape parallelogram. Among the 120 cells were from 5 to 15 cells which had not been closed combs or were empty. Of the 120, 100 closed cells were selected for measurement of hygienic behavior. On transparent film marked with dots 100 sealed cells. After 48 hours from the repositioning of the freeze-killed brood section, clean cells were counted. The colonies were hygienic if they completely removed an average of 95% of the frozen brood from both comb sections within 48 h. The assay, according to Spivak and Downey (1998), should be repeated at least twice.

Comparisons of hygienic behavior with the mite infestation rates (brood and population) of bees, and τhe strength of the colonies which were performed by using the statistical analysis software program SPSS 11.5 for Windows.

Results and Discussion

Variations in the expression of hygienic behavior

The hygienic behavior was studied in 23 colonies during 2011 and 2012. The results of this work show the large variation of hygienic behavior from month to month with a range of 10-70% in the first period and 1-73% in the second period (Tables 1 and 2). It was found that in five of the seven colonies in 2011 and in 12 of the 16 colonies in 2012 the coefficient of variation was high (CV > 15%). Also in 10 additional colonies, which were not part of the experiment, there were calculated in variation of hygienic behavior in October and November of 2012 (Table 3) after 7, 10 and 19 days between measurements. It showed a wide variation in the hygienic behavior, with a range of 16-111% and only one in ten colonies had little coefficient of variation (CV = 16%).

It has been reported in previous studies that the hygienic behavior is influenced by external environmental factors, as the abundance of nectar (Trump et al., 1967; Momot and Rothenbühler, 1971) and seasonal factors remain unclear (Mondragón et al., 2005) and it is not constant each year in each hive. Pernal et al. (2012) suggested this problem with the year-to-year comparisons of colonies chosen for breeding. While the analysis by comparing has been previously used to test the hypothesis that the selection increases hygienic behavior, it is limited in that comparisons among successively selected generations are made across different years (Palacio

Table 1
Percentage (%) of hygienic behavior within 48h in the period from May to August 2011

Year 2011						
Percentage (%) of hygienic behavior within 48h						
Colonies	May	June	July	August	Mean in 48h	CV % 48h
1	3	20	11	10	11^{a*}	63
2	99	82	72	100	88^{c}	15
3	13	40	26	35	29^{a}	42
4	50	26	24	5	26^{a}	70
5	31	30	37	12	28^{a}	39
6	30	75	78	50	58^{b}	39
7	65	74	74	83	74^{bc}	10
Mean					45	

* The comparison was made between the months for each hive separately. Based on the criterion of Duncan, means with different alphabet letter are statistically significant different in 0.05 level

Table 2
Percentage (%) of hygienic behavior in the period from May to July 2012

Year 2012					
Percentage (%) of hygienic behavior within 48h					
Colonies	May	June	July	Mean in 48h	CV % 48h
8	100	100	99	100^{d*}	1
9	97	86	85	90^{cd}	7
10	66	67	78	70^{bcd}	9
11	60	79	65	68^{bcd}	15
12	24	100	79	68^{bcd}	58
13	23	100	64	62^{abcd}	62
14	62	48	36	49^{abc}	27
15	16	62	67	48^{ab}	65
16	25	92	17	44^{abcd}	60
17	74	27	30	44^{ab}	60
18	20	38	72	43^{ab}	59
19	10	42	69	40^{ab}	68
20	15	23	78	38^{ab}	56
21	27	17	61	35^{ab}	66
22	25	24	45	31^{ab}	73
23	7	20	29	19^{a}	59
Mean				53	

* Same as Table 1.

Table 3
Repetition of hygienic behavior in 10 colonies

Year 2012						
Percentage (%) of hygienic behavior within 48h						
Colonies	17 Okt.	24 Okt	12 Nov	22 Nov.	MO 48h	CV % 48h
24	45	61	95		67	31
25	66	15	60	75	54	42
26	43	30	47	63	46	16
27	22	12	51	84	42	67
28	32	14	57	63	42	48
29	12	11	49		24	75
30	4	22	17		15	52
31	9	10	20		13	40
32	2	3	27		11	111
33	2	3	18		8	90

et al., 2000). In doing this, the year was confounded with generation, making it impossible to separate the variation from year to year due to additive genetic effects from the year-to-year environmental variation.

Unlike recent study by Bigio et al. (2013) showed that the availability of nectar (by feeding or not feeding sucrose syrup) not affects the expression of hygienic behavior and that hygienic behavior is not greatly affected by environmental conditions. In our work it was found that environmental conditions did not greatly affect the hygienic behavior at two colonies in the first period and at four colonies in the second period which had not large variations, as well it was found that there had not large variations in measurements per month (CV ≤ 15%).

According to previous studies (Arathi and Spivak, 2001) the bees with hygienic behavior can modulate the extent of performance of the behavior depending on the genetic composition of the colony. In colonies that were composed of 25% bees with hygienic behavior and 75% without hygienic behavior, hygienic bees responded to the stimulus of dead brood by increasing the rate of performance of hygienic tasks, by becoming more persistent and by extending the period of task performance inside the beehive, of middle-aged nurse bees, between 15 and 20 days old (Arathi et al., 2000). Genetically mixed colonies did not reach efficiency levels of a colony with 100% hygienic bees, suggesting that the proportion of bees in the colony that are able to perform the task at any time and must be large for successful colony-level expression of the trait. Increased activity of individual hygienic bees in mixed colonies is not sufficient to meet the increased demand for task performance (Arathi et al., 2006). Therefore, it is likely that the low variation in hygienic behavior of the six colonies in our experiment is due to the higher proportion of worker bees with the characteristic of hygienic behavior, which engaged with the cleaning of cells.

Correlation between hygienic behavior and infestation by the mite

The first period in seven colonies the degree of mite infestation in brood ranged from 0-24% (average 5%) and on the population of bees from 0-14% (average 3%). In the second period in 16 colonies the degree of mite infestation on the population of bees ranged from 0-17% (average 5%), (Table 4). Among the seven colonies there was no statistically significant difference in the degree of mite infestation in the population of bees (F = 1.389, df = 6, p > 0.05). Similarly, no statistically significant difference between the 16 colonies in degree of mite infestation in the population of bees (F = 1.426, df = 15, p > 0.05).

Between the hygienic behavior and the infection rate of mite on bee population it was found that there was no correlation between the two factors in 7 (r = 0.07, p > 0.05, N = 28), at 16 (r = 0.018, p > 0.05, N = 48) and a total of 23 colonies of two periods (r = 0.051, p > 0.05, N = 76) for a significance level of 0.05. In contrast to, the six colonies it was found that there was a positive correlation between the hygienic behavior and mite infestation on bee population (r = 0.568, p = 0.004, N = 20) for a significance level of 0.05.

It has been observed by Boecking and Spivak (1999) that most mites escape from the brood cells during the process of cleaning and are adherent on the nurse bees. According to the study of Arechavaleta-Velascoa and Guzmán-Novoa (2001) colonies with the highest mite population growth had more mites on adult bees, fewer mites falling to hive floors, and a lower proportion of injured mites. They concluded that a combined effect of grooming and hygienic behavior was responsible for the differences between colonies and found that grooming behavior explains 38% of the variation in the degree of mite infestation in colonies (Arechavaleta-Velascoa and Guzmán- Novoa, 2001). In our work we found that the hygienic behavior explains 32% of variation of infection rate of mite on bee population (R^2 = 0.323, p = 0.004, N = 20) for a significance level of 0.05.

In the first period, in the two colonies, the degree of mite infestation was on average 4.0% in the population of bees, while the second period, in the four colonies, the degree of infestation was lower at 3.3% on average. Noteworthy also is the fact that in the four colonies of the second period, the beehive with the less variation (CV = 1%) had a greater infestation on bee population (5.7% on average) compared to the hive with the largest variation (CV = 15%, 0.7% on the

Table 4
Average percentage (%) of mites' infestation in bee-brood and on bee population

Year	Number of colonies	Mite infestation, %							
		May		June		July		August	
		Br*	Pop**	Br	Pop	Br	Pop	Br	Pop
2011	7	1±1	2±2	3±3	1±1	4±4	2±2	11±9	7±6
2012	16		2±2		4±4		9±8		

*Br=brood, Pop**=Population of bees

average). It is therefore possible that differences in the degree of infestation of apiaries was due to different proportions of worker bees with the characteristic of hygienic behavior and grooming behavior, as well between different resistance mechanisms, these two behaviors are the most important.

Colonies reared for hygienic behavior are not possible to survive indefinitely without some sort of periodic treatment. However, it is encouraging that lines bred for hygienic behavior which may require less frequent treatments than unselected lines (Spivak and Reuter, 2001b). In our study we found that non-selected colonies for hygienic behavior to which no treatment was applied against the mite, during wintering, had more losses (collapsed five of the 12 original colonies, 42%), in contrast to colonies, selected for hygienic behavior, in which only one treatment was applied during the wintering (3 of the 19 colonies, 16%).

Correlation of hygienic behavior and the strength of the colony

The strength of the colonies was calculated from the number of frames of brood and bee population. In the first period, the 7 colonies had an average of 7 ± 2 frames of brood and 13 ± 7 frames of population, while the second period, the 16 colonies had an average of 7 ± 2 frames of brood and 10 ± 5 frames of population (Table 5).

At 7 colonies in 2011 it was found that between hygienic behavior and the number of frames of brood there was no correlation ($r = 0.186$, $p = 0.172$, $N = 28$). Similarly there was no correlation between the hygienic behavior and the number of frames of bees ($r = -0.090$, $p = 0.325$, $N = 28$) for a significance level of 0.05.

Also, from the statistical analysis of the 16 colonies in 2012 it was found that there was no correlation between the hygienic behavior and the number of frames of brood ($r = -0.176$, $p = 0.116$, $N = 48$) and the number of frames of bees ($r = -0.142$, $p = 0.169$, $N = 48$) for a significance level of 0.05.

The correlation between the hygienic behavior of six of the 23 colonies, which have a low coefficient of variability (CV ≤ 15%) and the number of frames of brood and population, also showed that there was no correlation between them ($r = 0.003$, $p = 0.495$, $N = 20$ and $r = -0.095$, $p = 0.346$, $N = 20$, respectively).

It has been shown by previous studies that weakened colonies with small bee populations have diminished response to hygienic behavior (Boecking and Drescher, 1993; Spivak and Gilliam, 1993). In a recent study (Bigio et al., 2013) it was observed that the manipulation of brood (by adding or removing brood) had no effect on levels of hygienic behavior.

Conclusion

In our study we found that the hygienic behavior is not linked to the strength of the beehive. Even in colonies with low variation in hygienic behavior, which had an average of 6 (± 2) frames of brood and 10 (± 4) frames of population, the change in strength of the colony did not affect the expression of the trait.

In conclusion it can be said that the selection of colonies with the trait of hygienic behavior should be based on the stability of repetitions of the measurements. At least three repetitions, either every month or the same month, will be taken in each hive for the final selection of colony with smaller variance (CV ≤ 15%). The hygienic behavior explains 32% of the variance of the mite infestation on bee population and with the grooming behavior may be sufficient as a mechanism of resistance to *V. destructor*.

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Table 5
Average number of frames of brood and bees

Year	Number of colonies	Number of frames							
		May		June		July		August	
		Br*	Pop**	Br	Pop	Br	Pop	Br	Pop
2011	7	5±1	7±2	7±2	15±2	8±2	17±3	6±2	14±3
2012	16	7±3	11±5	6±3	12±5	5±2	10±5		

*Br=brood, Pop**=Population of bees

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