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ORIGINAL ARTICLE

Expression of *Varroa* Sensitive Hygiene (VSH) in Iranian Honey Bees (*Apis mellifera* meda)

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ABSTRACT
*In Iran, honey bees cannot be kept without chemical treatments against Varroa destructor. The rich variety of native honeybee subspecies and ecotypes in Iran offers a good genetic resource for selection towards Varroa resistance. There are some examples of mite resistance that have developed because of natural selection in wild and managed Iranian populations. We tested five commercial sources of honey bees, Apis mellifera meda in East Azarbaijan province of Iran. Colonies from the five sources were measured for VSH and number of the fallen mites on the hive floor. The reduction of mite infestation in brood combs exposed to test colonies for 1 week differed significantly between groups. On average, colonies with natural low-level brood infestation reduced infestation by 26%. A positive correlation (Spearman coefficient) between number of fallen mites per 1000 bees (NFMB) with percentage of the mites that infested the brood (PMB) (r = 0.442**; p<0.01; n=50) was found. Results of path analysis showed %53 and %14 of variation (standard deviation) in NFMB are because of percentage of the mites in phoretic phase (PMP) and PMB variables respectively. This diversity offers rich potential genetic resources for selection on mite resistance.*
Key words: *Varroa resistance, Iranian honey bees, Varroa sensitive hygiene.*

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INTRODUCTION

Removal of infested brood is considered an important mechanism of resistance of the Asian honey bee *Apis cerana* Fabr. to the mite *Varroa jacobsoni* Oudemans [1-2]. Suppressed Mite Reproduction (SMR) is a trait of honey bees (*Apis mellifera* L.) that provides resistance to *Varroa destructor.* The mechanism of resistance in SMR bees is the removal of infested pupae from capped brood, so a better name is VSH (*Varroa* Sensitive Hygiene) bees [3]. Resistance to *Varroa* mites can help reduce or eliminate the use of other control methods by maintaining lower levels of *V.destructor* [4]. The expression of honey bee genetic resistance against *Varroa* mites may vary in different regions [5]. A number of honeybee stocks have been reported to exhibit resistance to *Varroa* mites, such as bees imported from Yugoslavia [6], the Primorsky Russian bee [7-8], suppression of mite reproduction lines [9] and the Africanized bees [10-11-12].

VSH bees uncap and remove infested brood and freed adult female mites usually transfer onto the bees removing the brood [13], but may eventually be free on the combs and expose to attack by bees. Thakur et al. (1997) documented that honey bees can detect, grab and bite free-moving mites [14].

In honey bee colonies VSH behavior of mature bees controls *Varroa* population [15]. SMR bees are able to detect mites laying in to the capped brood cells and kill them so appearance of these behavior of worker bees is due to the mite reproduction. In addition worker bees are not able to detect and remove sterile mites. So uncapping infested brood cells and removing mites is a selective behavior [16]. By evaluation and selection of honey bees for their VSH behavior it will be possible to reach *Varroa* resistant colonies. Also queens of these colonies will transfer *Varroa* resistance trait to her progeny by natural mating [17].

The VSH behavior of honey bees by indirect influence on length of phoretic stage stimulates grooming behavior and it is a basic mechanism in expression of other *Varroa* resistant traits [18]. It is crucial for a successful VSH bee breeding to use methods that are more convenient and selecting resistant colonies with at least 2 to 6 months field trials. By direct evaluation of VSH behavior of adult bees and selecting resistant colonies, rapid genetic gains are more expectable. One other quick way is to transfer brood comb from infested colonies to experimental colonies and study decreased infestation during 40 hours or one week [3]. There is a negative correlation between *Varroa* population growth and decreased *Varroa* reproduction during 48 hours and a week [19].

Varroa surviving bee (VSB) colonies were more efficient in removing mite-infested pupae from the cells, as reported in the MN Hygienic stock [20-21-22] and *Varroa* sensitive hygienic (VSH) bee strains from Louisiana [15]. Our goal was to breed honey bees resistant to diseases and parasitic mites to reduce the amount of antibiotics and pesticides used in bee colonies in Iran.

MATERIAL AND METHODS

The study was conducted at the apiary of Agriculture Education and Research Station of University of Tabriz from April through November 2014. The study started by preparing 60 colonies of honeybees from five regions (Tabriz=A, Azarshahr=G, Ahar=K, Bostanabad=N, Shabestar=S) of East Azarbaijan province. Of the 60 colonies, 10 were transferred to other location for breeding of *Varroa* mite. In this research, criteria of measuring hygienic behavior of bees against *Varroa* mites was the removal of mite-infested brood by bees, number of fallen mites per 1000 bees (NFMB) on the hives sticky bottom board and studying factors that put an effect on these variables. The *Varroa* sensitive hygienic (VSH) behavior of worker bees that removes infested pupae from capped brood was observed in 25 of 50 randomly selected colonies. In addition, number of fallen mites on the hives sticky bottom board was observed in 50 colonies. Before counting the NFMB, it is necessary to estimate *Varroa* population inside each hive. To do so, percentage of the mites in phoretic phase (PMP), percentage of the mites that infest the brood (PMB), adult bee population (ABP) and number of the bee brood (NBB) was estimated in experimental colonies.

Adult bee population estimates: The adult bee populations, was estimated visually by counting number of combs where both sides was covered with bees. As 1500 worker bees are enough to cover one side of a comb, it is enough to count number of combs whose both sides are covered with bees and then to multiply them in 3000 to calculate adult bee population in each colony. These figures calculated according to length and width of an adult bee's body and dimensions of a standard frame [23]. All categorical variables were transformed into continuous variables for statistical analysis.

Capped brood cell numbers estimates: Normally, each comb of honeybee has 7000 cells (3500 cells on each side of comb). In order for estimating number of capped brood cells, first the inner surface of one empty frame divided into ten equal parts by a thin metal wire. As each side of a comb include 3500-capped brood cells, each part of the wired empty frame includes 350 cells of waxy comb. Putting the wired frame on each comb of the experimental colony, number of inner parts of the wired frame filled with sealed brood was counted and multiplied in 350 to get number of capped brood cells.

Estimation of the mite percentage in Phoretic phase: In each experimental colony to estimate the percentage of the mites in phoretic phase about 150-200 adult bees was collected from the central brood frames of each hive into a jar with Chloroform. In the laboratory, after the bee samples and the mite stuck to their bodies anaesthetized with Chloroform, the separated mites and bees finally were count. Then, percentage of the mites in their phoretic phase was calculated.

Number of the fallen mites on the hive floor: In order to determine number of the fallen mites on the hive floor, first of all floors of the hives were set up with a 3 mm wire mesh in a wooden frame with a tray under it acting as a removable drawer. Then a white waxy paper smeared with white grease was put under that. Trapped mites on the sticky papers were counted once a week for 5 weeks. This period corresponds to approximately two mite generations (12 days in worker cells and approximately 5 days on adult bees for each generation) [24].

Removal of mite-infested brood by bees in experimental colonies: Hygienic responses of experimental colony bees towards mite infested pupae were investigated by transferring one comb of capped brood from a mite infested source colony (10 different sources) into each of 25 host colonies and monitoring hygienic responses that occurred during a week period. One comb of capped brood was removed from each colony before transferring the mite infested combs into the brood nest. The initial infestation rate (the sum of multiply and singly infested cells) of brood with *Varroa* mites in each comb, was found by sampling 100 capped brood cells in straight line transects on each side (50 cells per comb side) of comb. The initial infestation averaged 24.76 ± 2.61% (mean ± SE) for all combs that were used in this test (range, 3–49 %). The infestation rate after the test was estimated in the same way. The ratio of the number of infested pupae to the number of uninfested pupae was compared between the initial and

final samples to calculate the percentage decrease of all mite infested pupae from each combs [3]. Then, with regard to brood infestation rate, colonies of VSH group were divided to 3 subgroups as low (A), medium (B) and high (C). In these colony groups relation between VSH behavior of bees and brood infestation rate was investigated.

Statistical Analyses: Because the data obtained were nonparametric and did not provide the principle of variance analysis technique, to study multiple effect of the region factor, a multi comparison analysis was performed (Kruskal-Wallis test; Minitab V.16). Relation between variables of NFM, PMP, PMB, ABP and NBB estimated with Spearman correlation (SPSS V.18). In addition, Path Analysis was used to determine the effective factors on NFM.

For standardization of the worker bee population in colonies, the variable of NFM was varied to the number of fallen mites per 1000 bees (NFMB) and used these data in analyses.

RESULTS AND DISCUSSION

In a honey bee colony infested with *Varroa*, one of the main reasons of falling down the mites to the hive floor is hygienic behavior of bees. Hence, in this study in all colonies the number of fallen mites was counted for 5 weeks. A positive correlation (Spearman coefficient) between NFMB with PMP ($r = 0.314^{*}$; $p<0.05$; $n=50$) and PMB ($r = 0.442^{**}$; $p<0.01$; $n=50$) was found. In addition, a positive correlation was found between PMP and PMB ($r = 0.703^{*}$; $p<0.01$; $n=50$).

In order to specify the impact of PMP and PMB variables on NFMB and their interactions on each other, Path analysis was done and Determination coefficients were calculated (Fig 1). The path coefficients leading to NFMB were: 0.14 from PMP and 0.53 from PMB; and coefficient of

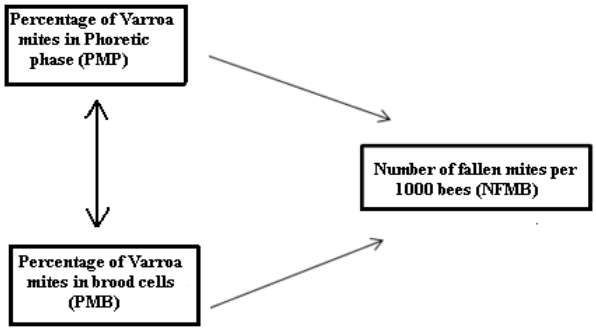

Fig1. Path analysis diagram of relation between the percentage of the fallen mites on the bottom board (NFMB) with percentage of *Varroa* in phoretic phase (PMP) and percentage of *Varroa* in brood cells (PMB).

determination that is the square of path coefficient, respectively was 0.019 and 0.281. The total effect of PMP and PMB on NFMB is 0.425. These results show that %53 and %14 of variation (standard deviation) in NFMB are because of PMP and PMB variables respectively.

According to these results, colonies of different regions were compared considering of the percentage of *Varroa* mite in phoretic stage. Multiple comparisons of Kruskal-Wallis and all pairwise comparisons of *p* values showed significant differences ($p<0.01$) in percentage of *Varroa* mite in phoretic stage between colonies of different apiculture regions (Table 1).

Table1. Descriptive statistics and Kruskal-Wallis multiple comparisons of percentage of *Varroa* mite in phoretic stage between different apiculture regions

Region	Mean ± SE	Min	Max	Rank Means
A	5 ± 1.1	2.3	14.7	31.1A
G	1 ± 0.4	0.0	4.3	12.3B
K	10 ± 2.5	0.0	23.1	34.6A
N	5 ± 1.5	0.6	16.9	27.2A
S	3 ± 0.8	0.6	7.0	22.3AB

DF = 4, P < 0.01

The other important factor that affects the number of mites falling onto the bottom board is the worker brood infestation level. In this study, relation between these two factor was investigated. Results based on Kruskal-Wallis test and pairwise comparisons of *p* values showed significant ($p < 0.05, 0.01$) differences between colonies of different region in terms of worker brood infestation level (Table 2). In addition, the

number of mites falling onto the bottom board is highly correlated (r = 0.442**; p<0.01; n=50) with the presence of brood infestation.

Table2. Descriptive statistics and Kruskal-Wallis multiple comparisons of worker brood infestation level between different apiculture regions

Region	Mean ± SE	Min	Max	Rank Means
A	6 ± 1.0	0	11	29.3A
G	2 ± 0.5	0	6	10.9B
K	12 ± 4.0	0	40	31.6A
N	9 ± 2.3	0	22	30A
S	6 ± 1.2	0	12	25.7A

DF = 4, P < 0.01

According to correlation coefficients estimated within regions, a high degree of correlation was found between percentage of brood infestation (PMB) and mites in phoretic phase (PMP) (Table 3).

Table3. Spearman correlation coefficients between PMB, PMP and NFMB within colonies of different regions

Variables		Regions				
		A	G	K	N	S
PMP	PMB	0.745*	-0.372	0.952**	0.954**	0.043
PMP	NFMB	0.427	0.176	0.539	0.491	-0.061
NFMB	PMB	0.279	-0.169	0.539	0.593*	0.202

A=Tabriz, G=Azarshahr, K=Ahar, N=Bostanabad, S=Shabestar; * P< 0.05 and ** P< 0.01

Among the numerous variables that were measured to assess VSH of bees, reducing the amount of brood infestation level, showed the highest coefficient of variation. Coefficient of variation of reducing the amount of brood infestation level (113.90 %) in comparison to coefficient variation of PMP (86.25 %), PMB (79.35 %) and NFMB (78.17%) showed the highest dispersion, and so is the best variable to evaluate hygienic behavior of bees against *V.destructor*.

Removal of mite-infested brood by bees was studied in 25 colonies. Then these colonies based on natural brood infestation level were divided into three low (A), medium (B) and high (C) subgroups (Table 4).

Comparison of the brood infestation level with removal of mite-infested brood showed that in the colonies with high hygienic behavior against *Varroa* (0.26 ± 0.045) there is low infestation level (0.01 ± 0.006); and in both desired variables significant differences (p < 0.05) were observed between groups.

Table4. Descriptive statistics and results of Kruskal-Wallis test on brood infestation level and removal of mite-infested brood in three subgroups of VSH examined colonies

Percentage of broods infestation,%					
Group	**Kolony No.**	**Mean ± SE**	**Min.**	**Max.**	**Means of Ranks**
A	9	1 ± 0.6	0.00	5	8.4 B
B	8	2 ± 0.4	0.00	4	12.1 B
C	8	5 ± 0.8	0.00	8	19.0 A
Reduction of *Varroa* infestation,%					
A	9	26 ± 4.5	0.01	0.46	18.6 A
B	8	7 ± 4.2	0.00	0.34	11.1 B
C	8	5 ± 4.2	0.00	0.34	8.7 B

SD = 2, P < 0.05; SD = 2, P < 0.05

CONCLUSION

In this study VSH behavior of honey bees was measured according to the decreased *Varroa* infestation in capped brood. The results of this study showed that VSH behavior of adult bees in low infested colonies was extremely high. In addition, among the group of VSH colonies that based on the rate of infestation were divided to three subgroups, significant differences ($P < 0.05$) were observed. Our results indicated that colonies more sensitive to *Varroa* mites has less infested pupas, as observed in previous studies [25-15-26-18-3-27-28-29] .

If mites in some bee colonies are phoretic for longer periods, they may have fewer chances of reproducing during their life and an increased potential for being groomed [30]. Selection for increased phoresy would enhance mite resistance. However, phoresy may be influence by other resistance traits. VSH reduces the number of mites in brood, and grooming reduces the number on adults. The mites released by VSH may either die or join the phoretic population, but in either case, the proportion of phoretic mites increases [31].

Results of our study confirmed previous ones and indicated that during uncapping and removing infested pupas, freed mites act as parasite for adult bees and lead to *Varroa* mites phoretic population growth ($r = -0.348$, $P < 0.05$). Hence, the measurement of pupae infestation rate and *Varroa* mites' phoretic population in different geographic regions colonies can be used as a criteria for evaluating bees resistance to *V.destructor*. Our results were similar to that of reported by Spivak [21].

The results of this study showed that in resistant honey bee colonies, brood infestation with *Varroa* mites is directly influenced by adult bees VSH behavior. Therefore, the estimation of brood infestation rate for a logical colony evaluation is suggested as an important criterion for mite resistance. Correspondingly, the possibility of breeding *Varroa* resistant lines in the Iranian honey bee (*Apis mellifera meda*) stocks was approved. Also mite-resisted honey bee breeding in Iran will retrieve Beekeepers some financial problems caused by *Varroa* infestation.

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