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Full Length Research Paper

Assessing hygienic behavior and attraction to *Varroa* mite (Acari: *Varroidae*) in Iranian honey bee (*Apis. mellifera meda*)

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In the current study, the hygienic behaviors of 5th instar larva of Iranian honeybees (*Apis mellifera. meda*) were investigated. The results of hygienic evaluation demonstrated that 35% of Iranian honeybees are hygienic. For more research, different levels of hygienic behaviors were used as a treatment and then the selected 5th instar larva were transferred to fundamental colonies with 10 to 12 % infestation. Three days later, the number of female *Varroa* in the capped cells were counted as attraction criteria for each treatment separately. The result demonstrated that hygienic colonies were fantastic because of their great interest for the least attraction. Moreover, the apparent correlation between resistant traits and performance traits exhibited that there are no significant relationship amongst resistance traits and performance traits. The relationship between uncapping and removing (which measured in the same recording day) was relatively high (>0.90). Furthermore, the correlation between hygienic behavior and attraction traits was negative so that the correlation between attraction and removing within 48 h after pouring liquid nitrogen was -0.86. The negative correlation between 2 major resistance traits (hygienic behavior and attraction traits) in the breeding stock deems that selection for resistance against *Varroa* mite and improved performance traits may be possible in the Iranian honeybees. Despite, it is believed that in the breeding stock for determining the best selection strategy, other resistant mechanisms must be evaluated.

Key words: *Varroa* mite*,* hygienic behavior (uncapping and removing), attraction, performance traits, Iranian honeybee.

INTRODUCTION

Varroa destructor is the most serious threat that the beekeeping industry faces worldwide (Anderson and Trueman, 2000). Nowadays, infested colonies are treated with chemical products which give a certain degree of mite control. However in the long term, using of miticides can cause a number of serious problems. For instance, mite populations are able to develop resistance to chemical products (Lodesani et al., 1995; Hillesheim et al., 1996; Elzen et al., 1999), miticides can leave chemical residues in the honey and wax (Faucon and Flamiini, 1990; Slabezki et al., 1991; Lodesani et al., 1992; Wallner, 1999) and treatments with chemical miticides increase the cost of honey production.

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The complete eradication of *V. destructor* is impossible. However, the beekeeping industry needs methods to maintain productive colonies with low levels of infestation. Regarding the serious disadvantages of chemical products, it is necessary to develop alternative methods to control *Varroa* mite. One option is breeding honey bee strains that are tolerant to *Varroa* mite. Those genotypes that are able to maintain low levels of mite infestation would allow beekeepers to keep healthier and productive colonies and would decrease the risks and costs associated by using chemical miticides. This goal, generating resistant honeybee genotypes, would be feasible if there is tolerance mechanisms against mite among honey bee populations, if there is variation for these mechanisms and if these mechanisms are heritable (Guzman-Novoa and Correa, 1996).

V. destructor is not a serious pest for its original host namely *Apis cerana* Fabr. This species of honey bee has developed tolerance mechanisms as a result of natural selection through a long association with the mite (Peng and Fang, 1987). In the case of *Apis mellifera*, some of the colonies survived in areas that were seriously damaged by *V. destructor*, which suggests the existence of a certain degree of tolerance in some genotypes of honey bees (Engels et al., 1986; Kulincevic and Rinderer, 1986; Moosbeckhofer et al., 1988; Wallner, 1999; Morse et al., 1991). One defense mechanism against the mite in *A. cerana* is grooming behavior. A worker bee is able to groom herself with her legs and mandibles to get rid of a mite. If worker bee cannot get rid of it, she performs a dance to attract other workers that may help her remove the mite from her body (Peng and Fang, 1987). Worker bees of *A. cerana* also have the ability to detect capped brood that is infested by *V. destructor*; the bees open infested cells to remove mites. This behavior mechanism is known as hygienic or removal behavior (Peng and Fang, 1987; Rath and Drescher, 1990; Boecking, 1992). The term, hygienic behavior, was coined by Rothenbuhler (1964) and refers to the genetic ability of honey bees within a colony to detect and remove diseased worker brood from the nest, thereby limiting disease transmission (Spivak and Gilliam, 1998a,b). Hygienic behavior involves two procedures where firstly worker bees uncap the wax-covered cells with diseased brood and secondly remove the damaged larvae or pupae. Hygienic bees most likely use olfactory cues to detect the abnormal brood (Masterman et al., 2001; Spivak et al., 2003). Hygienic behavior is one of several known mechanisms of resistance against *V. destructor* (Peng and Fang, 1987; Boecking and Spivak, 1999). Bees bred for hygienic behavior detect and remove worker brood infested with the parasitic mite, *V. destructor* (Spivak, 1996). Studies conducted by Boecking and Drescher (1991) showed that *A. mellifera* workers of the *Carniolan* race were able to detect, uncap, and remove pupae infested with *V. destructor*. Results of another study showed a negative correlation between the degree of hygienic behavior and the susceptibility to *V. destructor* of four honey bee lines (Buchler, 1992). In the United States, genetic programs to develop hygienic bee genotypes have been very successful (Rothenbuhler, 1964; Spivak, 1996; Spivak and Reuter, 1998a). Colonies selected for hygienic behavior had lower mite levels than non-hygienic ones. However, researchers point out that further experiments are necessary to determine to what extent hygienic behavior reduces the mite load within a colony (Spivak 1996; Spivak and Reuter 1998a; Boecking and Spivak, 1999). The relative attractiveness of worker brood and adult bees to the mite may be another tolerance mechanism (Buchler, 1989). Guzman-Novoa et al. (1996) found that the worker brood of Africanized bees was two times less attractive to *V. destructor* than the brood of European bees. Worker brood of hybrid bees (Africanized and European) was as attractive to the mite as brood of European bees. In the case of adult bees, European bees were more attractive to *V. destructor* than Africanized or hybrid workers. Other resistance mechanism is unknown physiological effect of either worker pupae or adult bees in some colonies that reduces mite reproduction (Camazine, 1986; Harbo and Hoopingarner, 1997; Harris and Harbo, 1999). In spite of the fact that the above mechanisms of mite tolerance have been identified in different populations of honey bees, the relative contribution of each of them to the overall mite tolerance is not clear. To develop a breeding program to select honey bee genotypes that restrain the growth of the mite population, it is important to determine if there is genetic variation for this trait and to determine the relative contribution of each of the mechanisms responsible for the tolerance. This information would facilitate the selection of honey bees based on the mechanisms that have a larger contribution to the mite tolerance. It is noticeable that *Varroa* mite made harsh damages in the Iranian honeybees during the summer of 1984. It is reported that all of the Iranian colonies are plagued by this parasite relentlessly. Therefore it is believed that breeding of Iranian honeybee against *Varroa* mite is mandatory.

The objectives of this study are: (1) To determine the situation of hygienic behavior and attraction traits in Iranian honeybee breeding stock (2) to assess relationship between genetic resistance mechanisms and performance traits (population and honey yield) and (3) to apply mass selection for surveyed genetic resistance mechanisms in the base population.

Figure 1. Hygienic behavior evaluation in the Iranian honey bee.

MATERIALS AND METHODS

Original breeding stock

The Iranian honeybee (*A. mellifera meda*) breeding stock were derived from a composite of Iranian honeybees existing in four provinces (Tehran, Markazi, Gazvin and Isfahan). In each generation, among 700 to 1000 colonies that were available in the Animal Science Research Institute of Iran, only 100 colonies that displayed relatively high performance (honey production), less swarming behavior and gentle temperament were propagated as breeding stock. In spite of appreciable research done on performance traits, they had not experienced any breeding practices for genetic resistance against disease and pests especially external parasite, *Varroa* mite, in the breeding stock. So, regarding the economic and welfare importance, the evaluation of Iranian honeybees for hygienic behavior against external parasites was undeniable.

Hygienic behavior

This assay, which was done in 250 colonies, provides an indirect measure for the ability of Iranian honey bee colonies to uncap and remove diseased and mite-infested brood. Colonies were tested for hygienic behavior by freezing a circular section of sealed worker brood containing 160 pupa cells within the comb by using liquid nitrogen (method described by Spivak and Reuter, 1998b). Within 3 days, the number of dead pupae that were in the process of being removed (were uncapped and / or partially removed), and the number completely removed from the cells were recorded each 24 h separately for trial colonies (Figure. 1). In the current study, only those colonies that were uncapped and removed more than 95% of the freeze-killed brood within 48 h were considered as hygienic. Forty eight hours (48 h) after pouring liquid nitrogen for hygienic behavior assessing, we noticed that there is the great phenotypic variation for hygienic behavior expression in the trial colonies.

Figure 2. Selection 5th instar larva and transformation 7 × 7 cm^2 compartments to pre-equipped frames.

Some colonies were capable of removing freezed and killed brood completely (hygienic) but certain not non hygienic.

Attraction

Brood attractiveness and its effect on the *V. destructor* reproductive capability were measured using a modification of the technique described by Guzman-Novoa et al. (1996). For evaluating the attraction, it was necessary to have some colonies sake experimental units. For selecting experimental units, approximately 100 adult workers were collected from a central comb containing open brood into a container containing hot water and detergent. In the lab, the mites were shaken off the bees through a strainer (De Jong et al., 1982). The number of mites per sample were counted and based on the weight of bees in each sample and a known weight of 100 wet bees from the same samples the number of mites per 100 bees were calculated and considered as a colony

Infestation

In the current research, 2 colonies with 10 to 12% infestation were selected as the experimental units. In the other steps, the removing rate at 48 h after pouring liquid nitrogen was used as a treatment because of its importance in the process of selection of hygienic colonies. In order to investigate the role of removing on the expression of attraction trait, five removing rate treatments were used. The treatments, 1, 2, 3, 4 and 5 were respectively, ≤60%, 60 to 70%, 70 to 80%, 80 to 90% and ≥90% removing rate 48 h after pouring liquid nitrogen. For the first three treatments, 3 colonies and for other treatments 4 colonies were considered as replication.

In the next procedure, 5th instars larva were cut from the trial colonies and transmitted to the pre-equipped frames which divided into 7 × 7 cm^2 compartments (Figure. 2). Afterward, these frames were placed in the center of selected experimental units. The infested colony was prepared in advance to receive the frame. Combs containing open brood cells were removed from the hive to increase the chances that *V. destructor* mites would infest the experimental brood. The queen of this colony was confined in a Benton cage to prevent her from laying on the experimental comb sections. Three days after introduction, the experimental frames were removed from the colony and the comb sections were placed in the individual plastic bags and kept at –5.0°C until they were analyzed. The cells of each comb section were uncapped and tested and the number of cells containing mites was recorded to infer brood attractiveness.

Figure 3. Measurement of adult bee population.

Colony evaluation criteria

Adult bee population

Adult worker bee population was visually determined by estimation of combs number covered by bees in each colony. The frames covered thoroughly in 2 sides by adult bees was considered as 1 frame whilst the less populous frames accounted for a fraction of 1 frame (Ibrahim and Spivak, 2006) (Figure. 3).

Brood population

The brood population was measured as follows: One prototype frame which was divided into 5×5 cm^2 compartments was used. By lodging this frame on the brood frames of each colony the compartments of the brood population was accounted for trial colonies (Tarpy and Page, 2001) (Figure. 4).

Honey production

Honey production was measured by weighing the boxes of honey as they were removed by the staff and subtracting the tare weight of the boxes from the total weight (Ibrahim and Spivak, 2006).

Experimental design

In order to investigate the effect of hygienic behavior on the attraction trait, an incomplete randomized design was applied. The model was as follow:

$$Y_{ij} = \mu + T_i + e_{ij}$$

In this model, Y_{ij} is the number of recorded mites in the 7×7 cm^2 area, μ is the population mean, T_i is treatment effect and e_{ij} is random error term.

The PROC GLM in SAS program was used for data analysis. A Duncan test was used for post-hoc comparison of the means. The relationship between two major resistance traits and the performance traits was conducted by using PROC CORR (JMP software, SAS 1994).

RESULTS

The hygienic behavior evaluation in 250 colonies showed that 35% of Iranian honeybees were hygienic and they afford to uncap and remove more than 95% of killed brood during 48 h after pouring liquid nitrogen (Figure. 5).

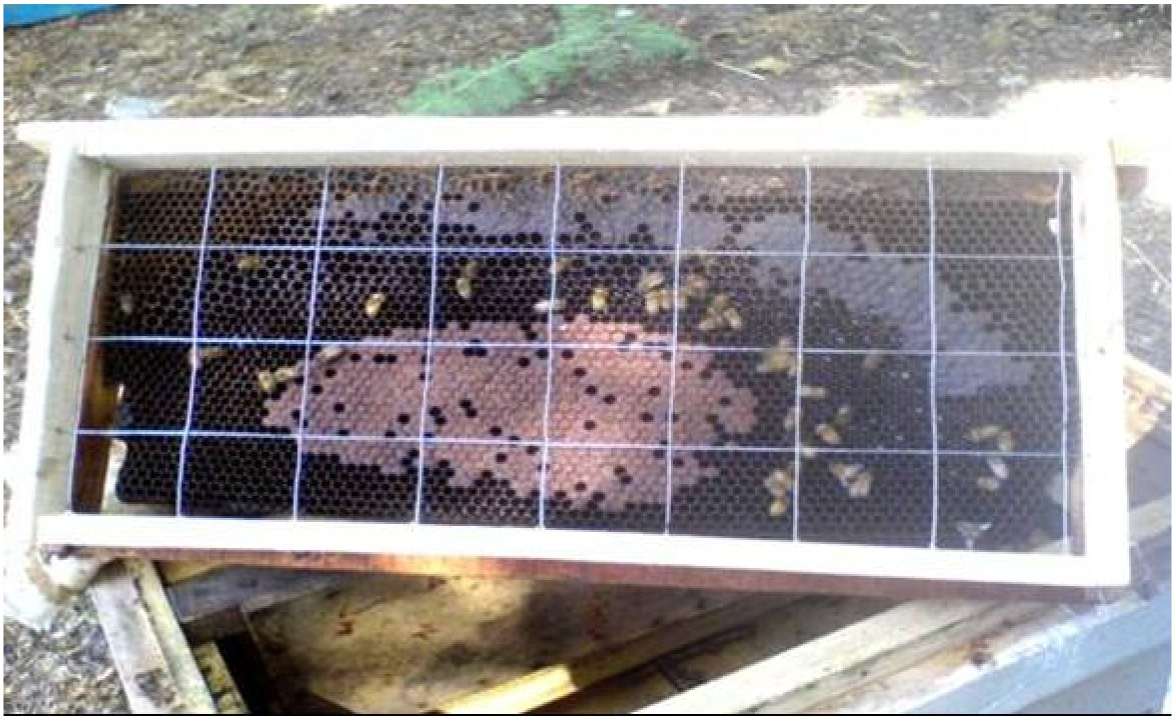

Figure 4. Measurement of brood population.

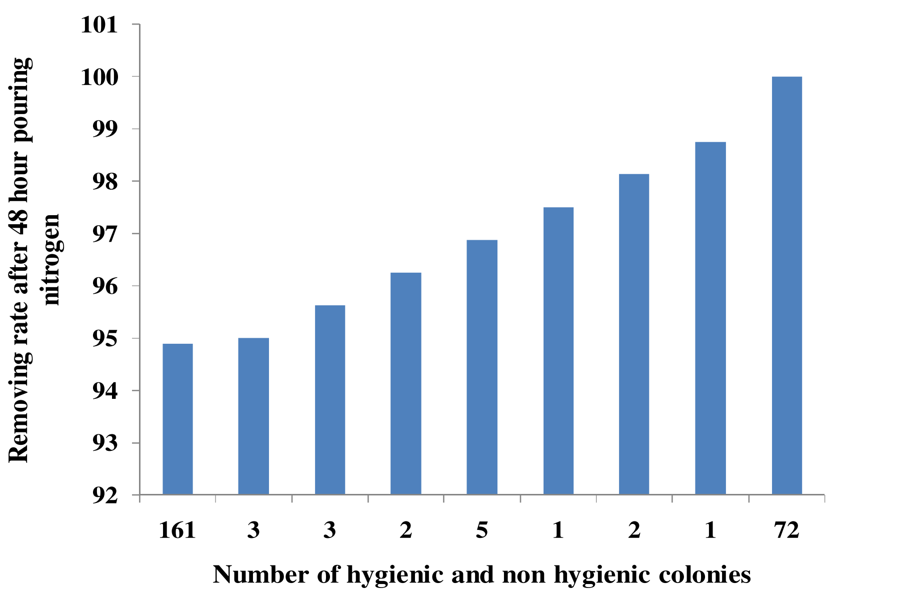

Figure 5. Number of hygienic (89) and non hygienic colonies(161) (X axis) with considering per cent of removing rate after 48 hour pouring liquid nitrogen(Y axis) in 250 colonies which were evaluated for hygienic behaviour.

Table1. Attractiveness of honey bee colony to *Varroa* mite.

Treatment	Removing rate (%)	Replication (colony)	Recorded removing rate (%)	Number of existed mite in the 7*7 compartment
1	≥60	1	13.13	35
		2	43.13	27
		3	49.38	25
2	60 - 70	1	68.13	26
		2	65.63	24
		3	67.5	25
3	70 - 80	1	78.75	24
		2	75	22
		3	79.38	23
4	80 - 90	1	86.88	10
		2	88.75	8
		3	89.38	3
		4	88.13	11
5	≥90	1	100	2
		2	100	2
		3	92.5	5
		4	90	6

From 250 colonies which were tested for hygienic behavior, 89 colonies were considered hygienic. Moreover, 72 colonies could afford to remove killed brood completely. One hundred and sixty one (161) colonies were spotted non hygienicl. The result of inscribed mite number in the brood cells of 7 × 7 cm^2 compartments for 5 treatments illustrated that by increasing the hygienic behavior, the attractiveness to *Varroa* mite declined in the colonies definitely (Table 1). In the first treatment, for colonies with 13/13 and 43/13% removing rate 48 h after pouring liquid nitrogen, 35 and 27 *Varroa* mites scrolled, respectively. Two *Varroa* mites for colonies with thoroughly removing rate (100%) has been noted similarly. Also, variance analysis demonstrated that hygienic behavior has significant effect on the declaration of attraction trait ($P < 0.0001$). Comparison of means exhibited that 3 first treatments, <80% removing rate 48 h after pouring liquid nitrogen, were lying in the same category whilst treatment 4 and 5 , >80% removing rate 48 h after pouring liquid nitrogen, were sitting in the other caste. Moreover, these 2 categories show significant differences between themselves (Figure. 6). The finding of apparent correlation showed that there were not any significant correlation between 2 major genetic resistance traits and performance traits (Table 2). There was observed moderate to high correlation between adult population with honey production (0.57) and brood population (0.88). Hygienic traits showed noticeable correlation (>0.6) between themselves in 3 consecutive days. Similarly, uncapping and removing which measured in the same recording day declared utmost correlation (>0.90). In this study, removing rate 48 h after pouring liquid nitrogen which is applied in selecting hygienic colonies exerted medium and high correlation with registered hygienic behavior in the first and third recording day, respectively. Remarkably, we deciphered negative correlation between hygienic behavior and attraction traits. Correlation between attraction and recorded hygienic behavior in the first recording day was insignificant (-0.4 and -0.45 with uncapping and removing). However, this correlation was significant with recorded hygienic behavior in the third day (-0.63 and -0.72 with uncapping and removing). Utmost negative correlation between 2 major resistance traits was found between removing rate 48 h after pouring liquid nitrogen and attraction (-0.86).

DISCUSSION

The results of freeze killed brood assay suggest that majority of Iranian colonies had 35% as hygienic and would not show clinical symptoms of chalkbrood (*Ascosphaera apis*) or American foulbrood (*Paenibacillus larvae*). Nowadays, in the developed countries for

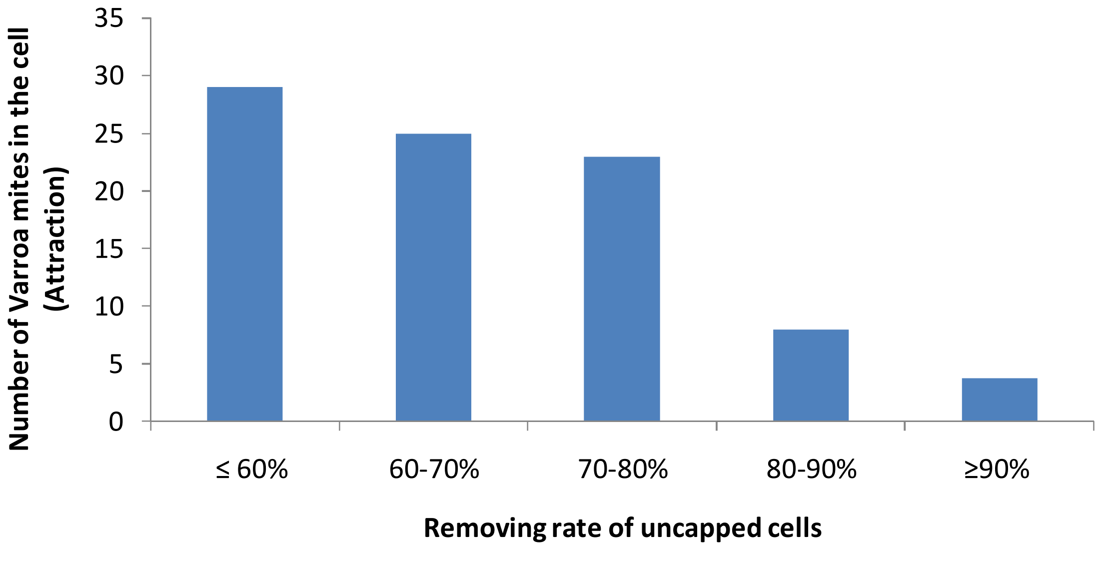

Figure 6. Larva attractiveness criteria in honeybee colonies with different hygienic behavior.

producing and introducing hygienic breeder queens to honeybee industry, colonies with removing rate more than 95% are selected as parents of next generation. With spotting the same evaluation criteria, our result showed that Iranian colonies with more resistant relevant reported hygienic behavior statue of other countries such as United States and Australia. Studies in the United State showed that 10% of colonies in nature are hygienic (Spivak and Downey, 1993; Boeking and Spivak, 1999). In the other scrutiny, Spivak and Garry (1998) reported that 10% of commercial colonies in the United States were hygienic. Also, in Australia, Oldroyd and Oxley (2008) demonstrated that hygienic behavior expression occurs in 20% of Australian honeybees. In addition, analysis of variance showed that expression of more hygienic behavior in the hygienic colonies eventuate the less attractiveness of brood larva. Probably, few number of *Varroa* mite in the hygienic colonies is the prudent reason for less attractiveness. Hygienic bees most likely use olfactory cues to detect the abnormal brood (Masterman et al., 2001; Spivak et al., 2003). In this situation, adult gravid mite chance for reproduction rate will be attenuated definitely. Ultimately, the number of entering *Varroa* mite to brood cell will be diminished in the next generation because of reduction in the *Varroa* mite population. Our result is in concurrent with the finding of other researchers. Guzman et al. (1996) in the comparison of hygienic behavior and attraction traits between 4 lines of Carniolan breed reported that amongst these lines Yugoslavian Carniolan which bred for hygienic behavior exerted the least attractiveness to *Varroa* mite. Moreover, Spivak and Gary (2001) in the widespread field trial declared that the number of *Varroa* mite in the brood and adult population of colonies which have been bred for hygienic behavior in the analogy with commercial colonies was the least. The finding of correlation demonstrated that there were not any significant correlation between resistant mechanisms and performance traits (Table 2). Our result is in pursuant with reported data by Spivak and Garry (2001). These researchers in the hygienic behavior survey between Italian hygienic line and non hygienic Starline hybrid did not announce any significant differences for adult and brood population. Moreover, moderate correlation between adult population with honey production (0.57) and brood population (0.88) was seen. Influence of adult population on honey production is clear. In the other words, populous colonies are strong enough to produce more honey before the nectar flow season. However, there is no significant correlation between honey production and brood population (0.45). Reported correlation is shownby the finding of Szabo and Lefkovitch (1989). These researchers recorded honey production, brood and adult population in the trial colonies for 2 years. Their assay showed that honey production was affected only by adult population significantly ($P < 0.05$); honey production not combined with brood

Table 2. Correlation between 2 resistance mechanisms and performance traits.

Variable	Brood population	Adult population	Uncapping-24	Uncapping-48	Uncapping-72	Removing-24	Removing-48	Removing-72	Attraction
Honey production	0.45 ns	0.57*	-0.15 ns	-0.3 ns	-0.31 ns	-0.1 ns	-0.24 ns	-0.19 ns	0.27 ns
Brood population		0.88**	-0.01 ns	-0.11 ns	0.13 ns	-0.03 ns	0.24 ns	0.22 ns	-0.19 ns
Adult population			-0.11 ns	0.15 ns	0.15 ns	-0.05 ns	0.31 ns	0.28 ns	-0.28 ns
Uncapping-24				0.7 ***	0.73**	0.95***	0.65 **	0.71 **	-0.4 ns
Uncapping-48					0.99 ***	0.66 ***	0.91 ***	0.93 ***	-0.66 ns
Uncapping-72						0.65 ***	0.91 ***	0.94 ***	-0.63 ***
Removing-24							0.63 ***	0.65 ***	-0.45 ns
Removing-48								0.94 ***	-0.86 ***
Removing-72									-0.72 ***

ns (non significant) (P>0. 05), ** significant (P<0. 01) and *** very significant (P<0. 001).

population. In this study, removing rate 48 h after pouring liquid nitrogen was applied in selecting hygienic colonies exerted medium and high correlation with registered hygienic behavior in the first and third recording day, respectively. Negative correlation between 2 major resistance mechanisms was related by the other researchers. Boecking and Drescher (1991) reported that Carniolan honeybees with more hygienic behavior indicated the least susceptibility to *Varroa* mite. In addition, Ibrahim and Spivak (2006) in their scrutiny of hygienic behavior and attractiveness between Minnesota hygienic line and SMR line related negative and significant correlation between attractiveness and removing rate 48 h after pouring liquid nitrogen (-0.61). Nonetheless, Miguel and Guzman-Novoa (2001) showed that there was positive and insignificant correlation between larva attractiveness and removing rate 48 h after pouring liquid nitrogen. Our finding shows that direct selection for hygienic behavior will lead to correlated response for attraction trait. In addition to selecting colonies that are hygienic and contain mites in worker brood with low reproductive success, it may be worthwhile to also select for another resistance traits, such as grooming behavior, which would limit the number of mites on adult bees (Ruttner and Hanel, 1992; Thakur et al., 1997; Arechavaleta-Velasco and Guzman-Novoa, 2001; Mondragon et al., 2005). Hence, it is hypothesized that in the Iranian honey bee, selection only for hygienic behavior because of the expression of hygienic behavior in several colonies and negative relationship between removing rate and attractiveness will be satisfactory in the oncoming years. However, it seems that in the breeding stock for determining the best breeding strategy against disease and pest, other resistance mechanisms such as grooming behavior, reproduction of *Varroa* mite in the larva and mummification of female *Varroa* in the wax, must be examined.

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