



RESEARCH ARTICLE - BEES

Hygienic Behaviors in the Iranian Honey Bee (*Apis mellifera meda*): Effects of Location and Season on Its Expression

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Abstract

The adverse effects of synthetic acaricides have led researchers and beekeepers to seek for the safe and sustainable alternatives to treat the honey bee varroosis caused by *Varroa* mite (*Varroa destructor*). The current research aimed to assess the expression of hygienic behaviors and the effects of season and location on its expression in 150 Iranian honey bee (*Apis mellifera meda*) colonies located in five cities of Kurdistan Province, Iran since 2023 to 2025. In addition, the correlation between hygienic behaviors and some functional-behavioral characteristics was investigated. The results revealed a significant difference 48 hours after pouring liquid nitrogen between the honey bee colonies of the studied cities regarding hygienic behaviors ($p < 0.05$). The means comparison of bees' hygienic behaviors showed that the highest and lowest average of hygienic behaviors were related to the colonies located in Saqqez (84.37%) and Qorveh (59.37%) cities, respectively. The mean of the total hygienic behaviors of the studied population 48 hours after pouring liquid nitrogen were 76.77%. This high percentage of expression of hygienic behaviors indicates that the hygienic behaviors in the honey bee population of Kurdistan Province of Iran are expressed to a favorable level. The results showed a positive correlation between hygienic behaviors with all the functional-behavioral characteristics of honey bee colonies in this research. Also, the effect of season on hygienic behaviors expression was significant ($p < 0.05$). In total, it can be concluded that the honey bee colonies of Kurdistan Province can defend themselves against the *Varroa* mite by performing hygienic behaviors. Therefore, it is possible to improve the level of these behaviors in the honey bee colonies of this province by implementing breeding programs.

Introduction

Honey bee (*Apis mellifera* L.) is one of the most important social insects in the world, which plays a prominent role in pollination and food security (Rahimi et al., 2016; Rahimi et al., 2023). Despite all the economic and social benefits of the beekeeping industry and the precious role of honey bees in nature, the honey bee colonies are currently affected by a series of unfavorable biotic and abiotic factors. *Varroa* mite (*Varroa destructor* Anderson & Trueman, 2000)

seems to be the main driver of the bee colony loss in most parts of the globe (Maleki Shireji et al., 2023). This parasite causes heavy economic damage to apiaries every year by feeding on the hemolymph and fat bodies of adult and brood bees and transmitting viral pathogens, especially the deformed wing virus (DWV) to its host (Traynor et al., 2020; Ramsey et al., 2021; Seltzer et al., 2022).

So far, numerous research studies have been conducted to control the *Varroa* mite throughout the world. Chemical compounds such as fluvalinate, flumethrin, coumaphos, etc.,



have been the first and most readily available options for treating *Varroa*-infested colonies. However, recent reports indicate that populations of this parasite have become resistant to the mentioned miticides (Sammataro & Avitabile, 2011; Dietemann et al., 2012). On the other hand, these compounds have been shown to pose harmful effects on human health, bees, and the environment (Căuia & Căuia, 2022). Hence, low-risk strategies are warranted for sustainable management of *Varroa* mites. Honey bees take advantage of a well-developed health-care system to rid themselves of any infections (Spivak & Reuter, 2001). Hygienic behaviors are one of honey bees' most important genetically based defense mechanisms against the *Varroa* mite (Spivak & Danka, 2021). Worker bees exhibit these behaviors to defend against honey bee pests and diseases, especially the *Varroa* mite (Masaquiza et al., 2021). This behavior includes detecting infested brood inside the capped cell, uncapping the cell, and removing the cell contents (Perez-Sato et al., 2009). It has shown that hygienic behaviors are controlled by genetic factors and by recessive alleles of two genes (Rothenbuhler, 1964; Wilkes & Oldroyd, 2004). In addition, the expression of hygiene behaviors is strongly influenced by environmental (temperature, humidity, nectar flow, etc.), chemical (pheromones, the smell of an infested pupa, the smell of ticks, etc.), and physical factors (movements, vibration, light, ...) as well as the mutual effects between all these factors (Gramacho & Gonçalves, 2009b; Rosenkranz et al., 2010; Meixner et al., 2015; Nganso et al., 2017). Colonies that remove more than 95% of dead, infected, and abnormal brood in less than 48 hours are considered hygienic. Typically, only about 10-12% of honey bee colonies in natural populations show desirable levels of hygienic behaviors (Perez-Sato et al., 2009). This proportion of hygienic colonies in honey bee populations can be increased through breeding, and many breeding projects have aimed to improve these bee traits (Amro & Amro, 2017). Since the hygiene colonies require less treatment than unhygienic ones, any reduction in miticide use will minimize operating costs for commercial beekeeping and reduce the risk of infection in colony products (Spivak & Reuter, 2001). It is believed that hygienic colonies produce more honey than unhygienic ones (Spivak & Gary, 1998; Spivak & Gilliam, 1998; Masaquiza et al., 2021). In a study, Banohosseini et al. (2012) investigated the hygienic behaviors of honey bee colonies in the breeding project of Iranian honey bees. They reported that the investigated colonies showed significant phenotypic diversity for hygienic behaviors. They also noted that the Iranian honey bee has a desirable genetic resistance against the *Varroa* mite. Tahmasebi et al. (2019) investigated hygienic behaviors and infestation levels in 100 Iranian honey bee colonies against *Varroa* mites. They found that hygienic colonies were 26% less susceptible to mite infestation than unhygienic ones. In another study, Taheri Imam Kandi et al. (2024) examined the hygienic behaviors of honey bee colonies in West Azerbaijan Province, Iran, and reported that the honey bee colonies in this province have desirable hygienic behaviors.

Given that the level of *Varroa* mite infestation varies among the colonies of an apiary or among the apiaries located in different regions, identifying the reasons for these variations is of great importance. Therefore, the present research aimed to evaluate the expression of hygienic behaviors and the effects of season and location on their expression in the Iranian honey bee population (*Apis mellifera meda*) in Kurdistan Province, Iran. This study also investigated the correlation between the level of hygienic behavior and some functional-behavioral characteristics of the bees.

Materials and Methods

Time and Location of the experiment and sample size

This study was conducted on the native honey bee (*Apis mellifera meda*) population of the Kurdistan Province of Iran during 2023-2025. This experiment was performed on honey bee colonies (colonies with non-sister queens) in five cities of Kurdistan Province (including Saqqez, Marivan, Sanandaj, Bijar, and Qorveh cities). In this way, two apiaries (apiaries with more than 300 colonies) from each city and 15 colonies from each apiary (colonies with more than 10% infestation) were randomly selected and examined. In total, this study was conducted on 150 native honey bee colonies (colonies with nine frames of bees) infected with *Varroa* mite for three years.

Experimental colonies

The colonies were homogenized in terms of queen age, population (adults and brood), and honey storage according to the instructions of Delaplane et al. (2005) before experimentation. During the experimental period, the studied colonies did not receive any acaricide. Also, the initial *Varroa* infection rate of the colonies was evaluated using the instructions of Dietemann et al. (2013) for adult bees and those of Zemene et al. (2013) for brood at the beginning of the experiment. The average initial infection rate of the colonies in the spring experiment was 9.94% with a range of 6 to 14.5%, and in the summer experiment, the average was 14.81% with a range of 8.24 to 17.71%. Then, hygienic and defensive behaviors, honey production, and population (brood and adults) traits of the studied bee colonies were evaluated for two consecutive years (2024 and 2025).

Evaluation of traits

a) Hygienic behaviors

The liquid nitrogen method was used to kill the pupa and evaluate the hygienic behaviors. The studied colonies were visited before the experiment. Then, each colony was given a frame coded to have pupae of the same age at the time the liquid nitrogen was used. Then, to stimulate the queens to lay, all the studied colonies were simultaneously fed (with sugar syrup 1 to 1) for three days. Then, the queen's oviposition area was marked on the frame of each colony.

Fifteen days after the queen laid eggs, instructions of Spivak & Reuter (1998) were used to kill the pupa and assess hygienic behaviors. According to this instruction, the number of dead pupa cells was counted and recorded 24 and 48 hours after pouring nitrogen twice daily (morning and afternoon). In this study, hygienic behavior assessment was done in spring and summer (June and July) of 2024 and 2025 (twice in each year), and the average was used in the analyses. According to the definition of a hygiene colony, colonies that removed more than 95% of dead pupa from their cells within 48 hours after pouring liquid nitrogen were considered hygiene colonies.

b) Honey production

The weight of the harvested honey and the remaining honey in each colony were calculated to evaluate the amount of honey produced by the colonies in the honey harvesting season in the studied cities. The amount of honey produced by each colony was obtained from the difference in the weight of each colony before and after honey extraction. The method of remaining honey level on the frames is used to estimate the exact weight of remaining honey in each colony. A special frame constructed from galvanized wire into 10 × 5 cm rectangles was used to evaluate the exact amount of remaining honey in each colony. Thus, each decimeter square of honey on both sides of the special frame was considered equal to 304 grams. In this method, the total of remaining honey in each colony was calculated by placing a special frame on both sides of the remaining honey frames in the colonies. Therefore, the total honey production of each colony in the year was obtained from the sum of the remaining honey and the harvested honey from each colony (Salehpor et al., 2023). The evaluation of this trait was also conducted during the honey harvest season in the region in 2024 and 2025, and its average was used in the analysis.

c) Defensive behavior

The evaluation of defensive behavior in the apiaries under study was done twice a year (June and July) using the theoretical method. In this method, honey bees were evaluated for behavior on the frame during colony visits and assigned a score accordingly. In this way, the assessment of defensive behavior according to bees' behavior was recorded for each colony as follows (Torkmandi et al., 2025):

Hives that were calm without smoking (score 4)

Hives that are calmed by smoking a little (score 3)

Hives that did not calm down with smoking average (score 2)

Hives that did not calm down Smoking a lot (score 1)

The evaluation of this trait was also done in 2024 and 2025, and its average was used in the analysis.

d) Population (adult and brood)

During the implementation of this project, the adult bee population was evaluated visually in two stages: the first

in June 2024 and the second in September 2024 and 2025. When visiting the colony, a frame covered with adult bees on both sides was considered a complete bee frame. If adult bees cover a part of the frame, a fraction of the frame is regarded as the population of adult bees. The total population of frames was considered the colony population and recorded for each colony. The brood population was evaluated in two stages, in June and September, in all the studied colonies. The brood population includes frames containing eggs, larvae, and pupae. Evaluation of the brood's population in June is similar to the assessment of the adult bees' population. In September, we used an empty frame cut into 5×5 cm squares to accurately calculate the brood population. The number of squares containing brood in each frame was counted by applying the special frame to every frame of brood in each colony. In this study, the brood coverage area was calculated in square centimeters (Tahmasebi et al., 2022).

Season effect

In this study, the hygienic behaviors of honey bee colonies in the studied cities were evaluated in June and July of 2023 and 2025 to investigate the effect of season on their expression, and the average was used for analysis.

Statistical analysis

Hygienic behaviors for all bees of each colony were calculated through the formula of Palacio et al. (2005) as follows:

$$\text{THB (Total Hygienic Behavior)} = \frac{X-Y-Z}{X} \times 100$$

THB = Colony Hygiene behaviors

X = The initial number of pupa cells (160 cells) treated with liquid nitrogen

Y = The number of dead pupa cells of the unremoved cap after 48 hours

Z = The number of dead pupa cells with uncapping cell caps but not removing cell contents after 48 hours.

In this study, the data were analyzed using an ANOVA based on a nested design by SAS V. 9.4 software. The comparison of means and the correlation between variables were performed using Duncan's test and Pearson's correlation method, respectively.

Results

Hygienic behaviors

Evaluation of hygienic behaviors in the spring and summer seasons (June and July months) of 2023-2025 on honey bee colonies of five cities of Kurdistan Province in Iran was performed 24 and 48 hours after pouring liquid nitrogen on combs with brood. In this research, a wide range of hygienic behaviors was observed during the experiment in the honey bee colonies studied. The results of the variance analysis of hygienic behaviors are presented in Table 1. The results of the variance analysis of hygienic behaviors 24 hours after

liquid nitrogen was poured showed no significant differences among honey bee colonies in the studied cities. Based on the results, a significant difference was observed in hygienic behavior between honey bee colonies from the studied cities 48 hours after liquid nitrogen was poured ($p < 0.05$). In this study, comparisons of means were conducted using Duncan's test and are presented in Table 2. The results showed that the highest and lowest levels of hygienic behavior 24 hours

after liquid nitrogen was poured were observed in honey bee colonies from Saqqez (61.29 %) and Qorveh (53.72 %), respectively. In 48 hours, honey bee colonies in Saqqez city, with an average of 84.37%, showed the highest hygienic behavior, while those in Qorveh city, with an average of 59.37%, showed the lowest. In total (monitored hours), the highest and lowest hygienic behavior were observed in honey bee colonies in Saqqez and Qorveh cities, respectively.

Table 1. Variance analysis of hygienic behaviors in honey bee colonies in the cities studied in Kurdistan Province, Iran.

Time (h)	Source of variations	df	Sum of squares	Mean of squares	F	P -Value
24	City	3	1652.22	431.251	2.141	0.081
	Apiary within the city	5	9441.129	193.129		
	Colony within the apiary of each city	144	85475.11	3851.175		
48	City	3	9971.752	3715.261	5.171	0.005
	Apiary within the city	5	25451.487	285.352		
	Colony within the apiary of each city	144	99861.895	6524.237		

Table 2. The comparison of the means of hygienic behaviors of honey bee colonies in the cities studied in Kurdistan Province, Iran.

City	The average hygienic behaviors of dead brood		Total of monitored hours*
	24 hours after pouring liquid nitrogen*	48 hours after pouring liquid nitrogen*	
Saqqez	61.29 ^a	84.37 ^a	88.25 ^a
Sanandaj	55.14 ^{ab}	68.14 ^{ab}	75.72 ^{ab}
Bijar	54.27 ^{ab}	56.76 ^{ab}	73.94 ^{ab}
Marivan	58.17 ^a	77.91 ^a	80.12 ^a
Qorveh	53.72 ^{ab}	59.37 ^b	65.85 ^c

*Different letters in columns indicate significant differences at $\alpha = 0.05$ based on Duncan's test.

Seasonal effect on the expression of hygienic behaviors of honey bee colonies

The comparison of means for the seasonal effect on the expression of hygienic behaviors 48 hours after liquid nitrogen was applied in the spring (June) and summer (July) seasons of 2024 and 2025 is presented in Table 3. The results showed that the season affected the expression levels of hygienic behaviors. Based on the results, the expression of these behaviors in the spring is significantly higher than in the summer ($p < 0.05$).

Table 3. The comparison of means effects of season on the expression of hygienic behaviors 48 hours after pouring liquid nitrogen in this study.

Trait	Month*		SE	p-Value
	June	July		
Hygienic behaviors	85.26 ^a	72.84 ^b	0.46	0.05

*Different letters in columns indicate significant differences at $\alpha = 0.05$ based on Duncan's Test. SE: Standard Error

Correlation of hygienic behaviors with functional-behavioral traits

Correlation analysis (Pearson) was used to obtain the relationship between the hygienic behaviors and functional-behavioral traits of honey bee colonies, and its results are presented in Table 4. The results showed a positive correlation between hygienic behaviors and all functional-behavioral traits of honey bee colonies in this study, with some correlations being significant. Based on the results, a positive and significant correlation was observed between the percentage of uncapped cell caps and the removal of cell contents 48 hours after liquid nitrogen was poured ($p < 0.01$). Also, a positive and significant correlation was observed between hygienic behaviors 48 hours after pouring liquid nitrogen and honey production ($p < 0.05$) and defensive behavior ($p < 0.01$).

Table 4. Correlation between hygienic behaviors of dead brood with the functional-behavioral traits of honey bee colonies in this study.

Traits	The uncapping of the cell cap (%) 48 hours after pouring nitrogen (1)	The removing the cell contents (%) 48 hours after pouring nitrogen (2)	Honey production (3)	Population (4)	Aggressive behavior (5)
1	1				
2	0.632**	1			
3	0.394**	0.215**	1		
4	0.198	0.063	0.417*	1	
5	0.56**	0.95**	0.037	0.098	1

*: Correlation at the 5% probability level. **: Correlation at the 1% probability level.

Discussion

Nowadays, the development of non-chemical methods, such as the use of resistant hygienic honey bee colonies against the *Varroa* mite, is being considered due to the adverse effects of chemical acaricides. Therefore, this study was conducted to investigate the expression of hygienic behaviors, their correlation with functional-behavioral characteristics, and the effects of season on the expression of hygienic behaviors in the Iranian honey bee population of Kurdistan Province in Iran. Results from various studies indicate that hygienic behaviors are expressed in the *Apis mellifera* species, and the expression of these behaviors varies across subspecies (Speak & Gray, 1998; Mondet et al., 2020). In this research, a wide range of hygiene behaviors were observed in the studied honey bee colonies. Based on the results, the average total hygiene behavior of the studied honey bee colonies 48 hours after liquid nitrogen was applied was 76.77%. This high level of hygienic behavior expression in the studied honey bee colonies indicates that it is favorable in the native honey bee population of Kurdistan Province, Iran. Several studies have been conducted across Iran on the hygienic behaviors of the Iranian honey bee, all of which have confirmed the favorable expression of these behaviors across populations. For example, we can refer to the studies by Elmi et al. (2007) on the honey bee population of West Azerbaijan Province, Banohosseini et al. (2013) on colonies of the Iranian honey bee breeding project, Tahmasabi et al. (2019) on the honey bee population of Alborz Province, Yahyaei & Karimi Dehkordi (2021) on the honey bee population of Lorestan province, Taheri Imam Kandi et al. (2024) on the honey bee population of West Azerbaijan Province, and Rahimi & Parichehreh (2024) on the honey bee population of Kermanshah Province in Iran. In some countries, such as Germany, Slovenia, Canada, and the United States of America, many studies have examined the hygienic behaviors of their native honey bee subspecies and reported varied results. Breeding programs in these countries to develop lines of resistant honey bees to pests and diseases, including the *Varroa* mite, have made significant progress. According to recent reports, some of

these countries have successfully created lines resistant to honey bees to the *Varroa* mite and some diseases, such as *American foulbrood*, by using hygienic colonies (Mondet et al., 2020; Sainsbury et al., 2022).

Based on the results of this study, 8% of the colonies (12) exhibited very favorable hygienic behavior. Tahmasebi et al. (2019) conducted a two-stage study on the hygienic behaviors of the honey bee population in Alborz Province, Iran. These researchers reported that 21% of the studied colonies in the first stage and 18% in the second stage had favorable hygienic behaviors. In another study, the expression of these behaviors was reported in 26% of the studied honey bee colonies at a favorable level (Speak & Gray, 1998). Also, Kekecoglu et al. (2015) investigated the hygienic behaviors of 250 *Apis mellifera anatoliaca* colonies using the liquid nitrogen method. These researchers reported that five colonies exhibited superior hygienic behavior among the colonies studied. Based on the results of this study and other studies on other Iranian honey bee populations, it can be concluded that the hygienic behaviors in Iranian honey bees are expressed at a high level and have a favorable ability and superiority in terms of hygienic behaviors over other honey bee subspecies in the world. Also, the expression levels of these behaviors differ across populations of Iranian honey bees in different regions of Iran. The results of the present study, in agreement with previous studies on Iranian honey bees, indicated that hygienic behaviors are expressed at a favorable level in the honey bee's native population in the Kurdistan Province of Iran. Therefore, breeding the native honey bee population in this province can yield valuable results by creating colonies resistant to *Varroa* mites in the future.

The results of this study showed that hygienic behaviors are affected by season, with expression in spring significantly higher than in summer ($p < 0.05$). This difference can be attributed to increased nectar entering the colony and a larger colony population in spring. Various studies have already reported this. For example, we can refer to the studies of Tahmasebi et al. (2019), Yahyaei & Karimi Dehkordi (2021), and Taheri Imam Kandi et al. (2024). This subject has also been investigated by other researchers on other honey bee subspecies. Gramacho and Gonçalves (2009a) and Snyder et al. (2024) reported that the hygienic behaviors of honey

bees are influenced by various factors, including genetics, nervous systems, colony size, and social and environmental conditions, and these factors can influence the expression of hygienic behaviors in colonies. Bigio et al. (2013) investigated the effect of season on the expression of hygienic behaviors. These researchers reported that seasonal factors affect the expression of hygienic behaviors. Due to the larger population size of the colony, more larvae in the colony in the spring season, and more irritability in the colony, the number of hygienic worker bees is higher and faster show hygienic behaviors in the spring than in the summer season. Genetic factors also play a major role in the expression of hygienic behaviors. Oxley et al. (2010) estimated the heritability of hygienic behaviors to be 0.65. These researchers reported that the expression of these behaviors varies across honey bee species and subspecies.

The correlation analysis showed a positive correlation between hygienic behaviors and all functional-behavioral traits in honey bee colonies. Based on the results, a positive and significant correlation was observed between the percentage of uncapped cell caps and the removal of cell contents 48 hours after liquid nitrogen was poured ($p < 0.01$). The positive correlation between uncapping the cell cap and removing cell contents is obvious. Because the possibility of removing cell contents increases with the increase in the uncapping of the cell cap and pupae availability. Similar results were also reported for a positive and significant correlation between the uncapping of the cell cap and with removing of cell contents in other Iranian honey bee populations by Banohosseini et al. (2013), Mansouri Zalati et al. (2017), Tahmasabi et al. (2019), Yahyaei & Karimi Dehkordi (2021), Rahimi & Parichehreh (2024), and Taheri Imam Kandi et al. (2024). A positive and significant correlation was observed between hygienic behaviors 48 hours after pouring liquid nitrogen and honey production, as well as defensive behavior. These findings were consistent with the results of studies by Banohosseini et al. (2013), Mansouri Zalati et al. (2017), Yahyaei & Karimi Dehkordi (2021), and Masaquiza et al. (2021). Rothenbuhler et al. (1964) showed that defensive and hygienic behaviors have separate inheritance, and colonies with higher hygienic behaviors are as defensive as other colonies. Therefore, the results of Rothenbuhler et al. (1964) are not consistent with the results of the present study. The interaction of genotype and environment influences honey bee colonies. According to the results of the studies, the genetic correlation is reduced in most cases, and different environmental factors such as temperature, humidity, season, nectar flow, population size and colony abilities affect the hygienic behaviour of honey bee colonies (Spivak & Giliam, 1993; Boecking & Spivak, 1999; Gramaco et al., 2009). Finally, our findings showed that hygienic behaviors are expressed at a favorable level in the honey bee's population native to the Kurdistan Province in Iran. Therefore, it is possible to improve the rate of these behaviors in the honey bee population of this province by implementing long-term breeding programs. Also, created

a honey bee line resistant to *Varroa* mite by using hygienic colonies. Also, further investigation and research on other honey bee hygienic behaviors, such as *Varroa* sensitive hygiene (VSH), grooming, uncapping & recapping hygienic behaviors, and other honey bee behavioral mechanisms against *Varroa* mite in the honey bee population of Kurdistan Province is suggested to identify the hygienic colonies and create hygienic lines resistant to *Varroa* mite.

Authors' Contribution

A.R.: Conceptualization, methodology, software, formal analysis, validation, investigation, data curation, writing-original draft, writing-review & editing, project administration.

S.P.: Methodology, validation, supervision.

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