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# A Comparative Assessment of Hygienic Behavior of Carniolan (*Apis mellifera carnica* Pollmann) and Yemeni (*Apis mellifera jemenitica* Ruttner) Honeybees Using Infra-Red Photography Video Recording

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**Abstract:** The use of infra-red photography video recording is very useful for conducting behavioristic studies of honeybees against many brood diseases. The removal of dead or diseased brood from capped cells by honeybee workers is a heritable trait that confers colony-level resistance. This work aimed to compare the hygienic behavior of the native (Yemeni bees, *A. mellifera jemenitica*) and the exotic (Carniolan bees, *A. m. carnica*) honeybee races in Saudi Arabia using an infra-red photography video recording. In addition, hygienic behavior towards the related and non-related combs was examined. Therefore, it is possible to obtain honeybee colonies with greater disease resistance. The pin-killing method and infra-red photography video recording were used for the evaluation of hygienic behavior in colonies of the two races. Significant differences in hygienic behavior between the two races were detected at the beginning of the experiment. Under the environmental conditions of eastern Saudi Arabia, the Yemeni honeybee colonies showed a higher number of uncapped and cleaned cells containing dead brood in either the brood comb from the same colony, or the brood comb from the same race but a different colony, or brood comb from a different race. It was concluded that the honeybee's ability to detect and clean the dead brood from comb cells can be correlated with race and it is more efficient for the non-related individuals of the same race than from a related or another race. The outstanding performance of a few individuals in the expression of various traits indicates their usefulness in carrying out breeding programs for Varroa resistance.

**Keywords:** honeybee; hygienic behavior; infra-red photography; pin-killing method; Varroa resistance



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## 1. Introduction

Varroa mite is an ectoparasite that infests brood and adult honeybees. The rate of varroa infestation in bee colonies varies according to factors such as bee race [1–3], individual sex [4,5], comb age [6], and comb cell size [7,8].

Hygienic behavior is one of many indicators of bee resistance to diseases. Therefore, it is useful to assess this trait [9–11]. In the honeybee (*Apis mellifera* L.), some bee workers are capable of recognizing and removing diseased, damaged, or dead brood in capped brood cells. This uncapped and removal behavior is termed hygienic behavior [12], which is also a mechanism of disease resistance if bees can remove brood from the nest before the pathogen becomes infectious. This behavior makes colonies resistant to the American foulbrood [13–16], chalkbrood [14,15,17], and the parasitic mite, *Varroa destructor* [3,18–22]. The hygienic behavior of honeybees has been described as a two-step process: bees uncap wax-covered cells containing diseased brood (fifth instar larvae and pupae) and then remove the brood.

The efficiency of hygienic behavior is affected by the ability of worker bees to recognize bacteria, fungi, virus-infected brood, or Varroa-infested brood (by chemical and physical cues) [23]. The behavior of the bees is influenced by external stimuli that can be detected by sensory organs. One of these organs, the antenna, is highly complex with a series of sensory components, including the plate organs (sensilla placodea), which are used for the perception of odors [24]. In Egypt, colony strength and assay type have been shown to have significant effects on the expression of hygienic behavior at 6, 24, and 48 h. In addition, their interactions at 6 and 48 h were significant [25]. Furthermore, Zhao et al. [26] studied the behavioral responses of *A. mellifera* adult workers to odors from the healthy brood and diseased brood; they found that the adult workers of some colonies have olfactory perception sensitive enough to detect infected brood.

In general, hygienic behavior seems to be highly variable [27,28], which may be an advantage for selective breeding. The defensive behavior has been studied in different honeybee races and hybrids, e.g., *A. melliera carnica*, *A. m. caucasica*, *A. m. intermissa*, *A. m. lamarckii*, *A. m. ligustica*, *A. m. meda*, *A. m. syriaca*, and the dwarf honeybee, *A. florea* in Algeria [2,29,30]. Pereira et al. [31] concluded that the type of comb and time of day should be taken into consideration when hygienic behavior is evaluated in honeybees.

Palacio et al. [32] studied the activities involved in the hygienic behavior of individually tagged bees from selected hygienic and non-hygienic colonies in the presence of a chalkbrood-infected brood (*Ascosphaera apis*) or pin-killed brood. Bigio [33] stated that both beekeeping and applied honeybee research benefits from stocks of bees that display particular traits and desirable qualities. Many traits of interest in beekeeping, such as honey production [34,35], wax production, defensive [36,37], and hygienic behavior [38–41], are heritable and arise from the behavior of the workers. Selective breeding may be carried out, since hygienic behavior has a high heritability [38–41].

In the breeding programs, the defensive behavior of the honeybee is usually assessed subjectively [36]. Based on Dziechciarz et al. [42], the dead brood removal was more efficient from small-cell combs than from standard-cell combs; therefore, we may hypothesize that the Yemeni honeybees have a higher ability for cleaning dead brood from comb cells than the Carniolan bees. The main objective of this work was to understand the defense mechanisms of the hygienic behavior of the native (Yemeni bees, *A. m. jemenitica* Ruttner) and the exotic (Carniolan bees, *A. m. carnica* Pollmann) honeybee races. In addition, we compared hygienic behavior in related individuals and in non-related ones. It is therefore possible to obtain honeybee colonies with greater disease resistance by selecting the colonies from which to breed from those that show higher levels of hygienic behavior.

## 2. Materials and Methods

### *2.1. Site of the Experiment*

The study was performed at the apiary of the Training and Research Station, King Faisal University, in Al-Ahsa province (25°25′46″ N, 49°37′19″ E; 121 m above sea level) in eastern Saudi Arabia, September 2021.

### *2.2. Treatments*

In this experiment, two groups of colonies were housed in Langstroth hive boxes with nine frames. Each colony has a laying queen, worker bees, stores of honey and pollen, and four frames of brood at all stages. The first group (12 colonies) was headed by queens of the native race (Yemeni bees, *A. mellifera jemenitica* Ruttner), and the second group (12 colonies) with queens of the Carniolan (*A. m. carnica* Pollmann) race obtained from the Institute for Bee Research, Hohen Neuendorf, Germany. Each group was divided into three subgroups according to the pin-killed brood comb, as follows: (1) Yemeni colony and the pin-killed brood comb from the same colony ($T_1$), (2) Yemeni colony and the pin-killed brood comb from another colony of the Yemeni bees ($T_2$), (3) Yemeni colony and the pin-killed brood comb from a Carniolan bee colony ($T_3$), (4) Carniolan colony and the pin-killed brood comb from the same colony ($T_4$), (5) Carniolan colony and the pin-killed brood comb from

another colony of the Carniolan bees ($T_5$), and (6) Carniolan colony and the pin-killed brood comb from a Yemeni bee colony ($T_6$)

### *2.3. Experimental Procedure*

According to Gramacho et al. [43], the pin-killed brood assay was used to detect which race showed more hygienic behaviors toward pin-killed brood between related or unrelated races. The combs were observed by using infrared photography video recording continuously for 3 days in each experiment [44] using a glass-walled observation unit (Figure 1).

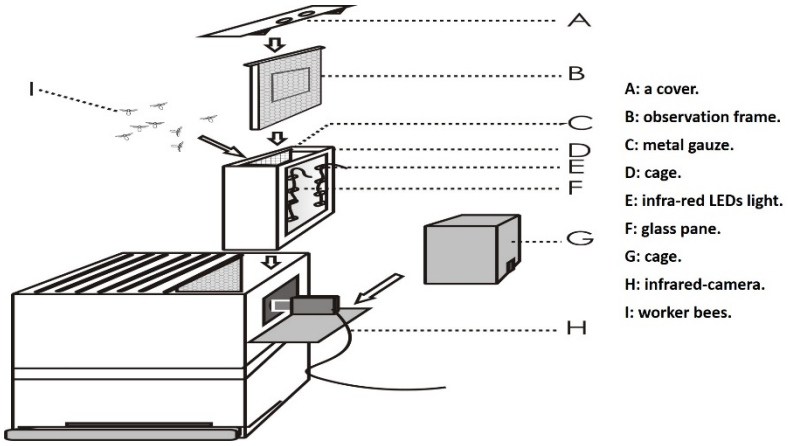


**Figure 1.** An illustration of the observation unit components.

The behavior was recorded using a camera sensitive to the infrared portion of the spectrum (Panasonic WV-NP1004 megapixel color network IP). Infrared LEDs (OSA Opto-Light, Berlin, Germany, Type: OIS 330 880) were used for illumination; because bees do not respond to this wavelength of light (880 ± 10 nm) [45], they were not disturbed by this illumination during the long observation periods [46]. The acquired image sequences were stored on a digital long-term videotape recorder (Panasonic, DVD, model DMR-E500H, Kadoma, Japan). A frame cage with a sliding panel of glass on one side and metal gauze on the opposite side was used. The cage contained a brood frame with both sealed and unsealed brood, honey and pollen. The eight-frame bee colony was kept in a polystyrene beehive and the caged brood frame was inserted with the gauze facing the rest of the colony (allowing contact), and the glass panel was placed against the wall of the beehive. Through a small hole in this wall (sealed off from normal light), observations could be made with an infrared video camera (with a black/white CD chip). The experiments were repeated four times per comb/race.

A comb containing capped worker brood cells aged 10 to 12-days-old was taken from each colony. One side of the comb area (5 cm × 6 cm) included 100 capped worker brood cells, each delimited. We performed reciprocal transfers and exchanges of capping worker brood (pupae) between colonies. In the pin-killed assay, brood was killed using an entomological pin from the front of the comb. The behavior of individual bees is documented with the infrared camera located within the camera unit facing the observation unit. Each time the honeybee workers performed an activity on the cells containing mummies, the following data were recorded: (1) number of workers arrived at the cell and (2) activity performed (uncapped or removal). After 3 days, the recorded data were inspected as follows: the number of workers that arrived at the cell during one hour and the percentage of the cleaned cells containing dead brood were counted at 1, 2, 4, 6, 8, 8, 10, and 12 h. The inspection was finished at 12th hour because 100% of cells containing dead brood were cleaned.

### *2.4. Statistical Analysis*

The experiment was designed in a factorial (2 × 3) completely randomized design. The data were analyzed using the two-way analysis of variance (ANOVA). The normality in the data was tested by the Shapiro–Wilk normality test, which indicated the normal distribution of the data. Therefore, the original data were analyzed. The ANOVA was used to assess differences between the two honeybee races tested via the PROC GLM function in SAS version 9.1 [47]. The means of the Yemeni and Carniolan bees were compared using Tukey's HSD post hoc test. Pearson's correlation coefficients between the number of workers that arrived at the dead brood cells and cleaned cells were determined.

## 3. Results

The data illustrated in Figure 2 show that the number of workers arrived at the dead brood cells at the first hour of the experiment in Yemeni bee colonies vs. the Carniolan bee colonies was 8.00 vs. 3.00 workers/100 cells when the brood combs were from the same colony, 5.00 vs. 5.00 workers/100 cells when the brood combs were from the same race but different colonies, and 10.00 vs. 6.00 workers/100 cells when the brood combs were from a different race; at the second hour, the number of workers reached 20.00 vs. 5.00 workers/100 cells, 20.00 vs. 15.00 workers/100 cells, and 25.00 vs. 10.00 workers/100 cells, respectively. At the fourth hour after killing the larvae in the cells, the number of workers that arrived at the dead brood cells were 35.00 and 10.00 workers when the brood combs were from the same colony, 40.00 and 30.00 workers/100 cells when the brood combs were from the same race but different colonies, and 45.00 and 15.00 workers/100 cells when the brood combs were from a different race in Yemeni and Carniolan colonies, respectively. In the sixth hour, the number of workers increased to 50.00 vs. 25.00 workers/100 cells, 45.00 vs. 35.00 workers/100 cells, and 40.00 vs. 20.00 workers/100 cells, respectively, and at the eight hour reached 70.00 vs. 63.00 workers/100 cells, 60.00 vs. 55.00 workers/100 cells, and 65.00 vs. 30.00 workers/100 cells, respectively. In the tenth hour, the number of workers decreased to 13.00 vs. 16.00 workers/100 cells, 38.00 vs. 57.00 workers/100 cells, and 55.00 vs. 54.00 workers/100 cells, respectively, and at the twelfth hour reached 28.00 vs. 15.00 workers/100 cells, 43.00 vs. 22.00 workers/100 cells, and 39.00 vs. 18.00 workers/100 cells, respectively.

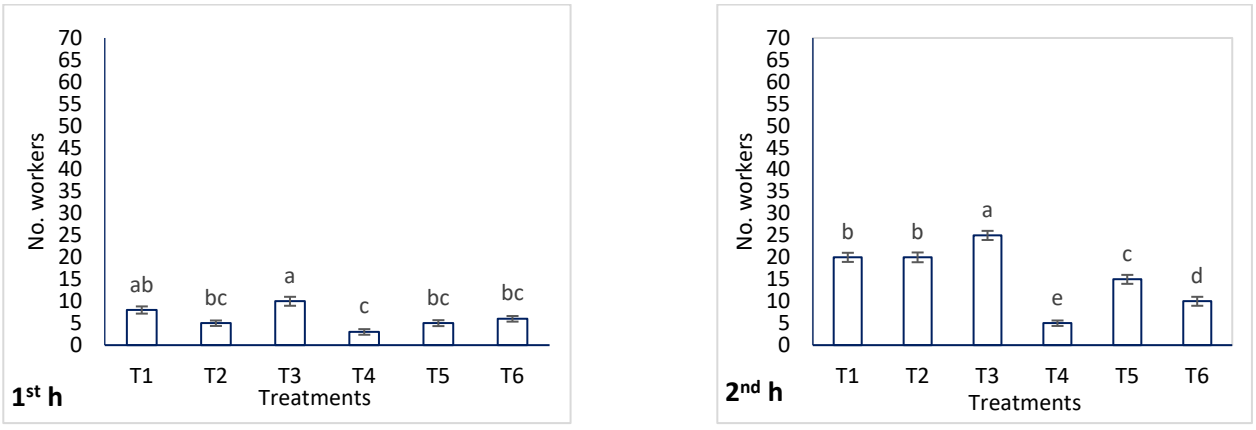


**Figure 2.** *Cont.*

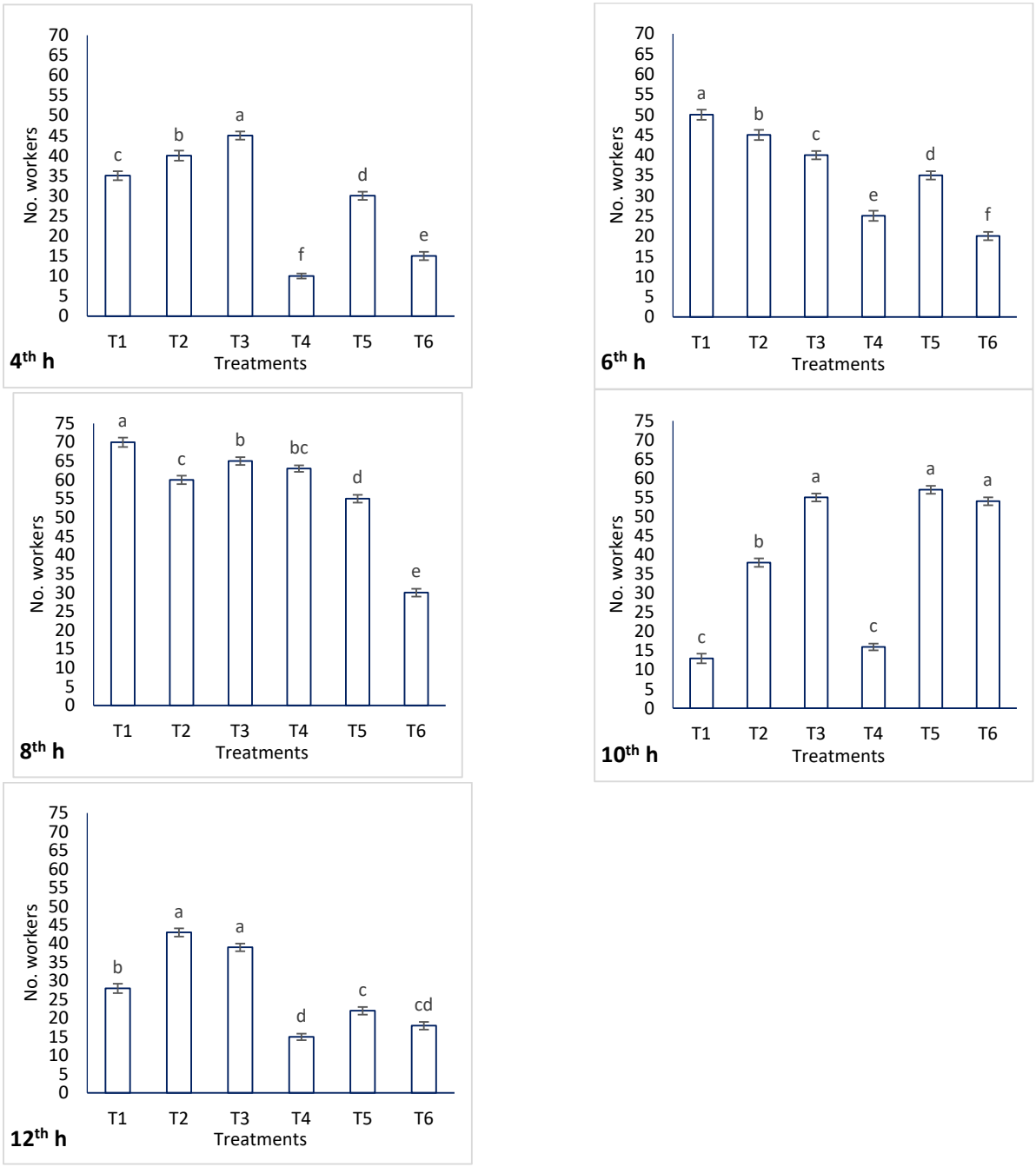


**Figure 2.** Mean number of workers arrived at 100 cells with dead brood in Yemeni and Carniolan honeybee colonies. Different letters indicate significant differences at the 0.05 level. $T_1$ = Yemeni colony and the pin-killed brood comb from the same colony, $T_2$ = Yemeni colony and the pin-killed brood comb from another colony of the Yemeni bees, $T_3$ = Yemeni colony and the pin-killed brood comb from a Carniolan bee colony, $T_4$ = Carniolan colony and the pin-killed brood comb from the same colony, $T_5$ = Carniolan colony and the pin-killed brood comb from another colony of the Carniolan bees, and $T_6$ = Carniolan colony and the pin-killed brood comb from a Yemeni bee colony.

As shown in Figure 3, the percentage of the cleaned cells containing dead brood at the second hour of the experiment, the Yemeni bee colonies vs. the Carniolan bee colonies was 5.00 vs. 2.00% when the brood combs were from the same colony, 13.00 vs. 4.00% when the brood combs were from the same race but different colonies, and 12.00 vs. 3.00% when the brood combs were from a different race; at the fourth hour, the ratio of the cleaned cells reached 16.00 vs. 12.00%, 48.00 vs. 19.00%, and 37.00 vs. 8.00%, respectively; at sixth

hour reached 38.00 vs. 32.00%, 72.00 vs. 42.00%, and 48.00 vs. 26.00%, respectively; at the eighth hour, the ratio of the cleaned cells reached 87.00 vs. 67.00%, 90.00 vs. 83.00%, and 70.00 vs. 59.00%, respectively. At the tenth hour of the experiment, the Yemeni bee colonies vs. the Carniolan bee colonies was 95.00 vs. 77.00% when the brood combs were from the same colony, 96.00 vs. 89.00% when the brood combs were from the same race but different colonies, and 88.00 vs. 86.00% when the brood combs were from a different race. At the twelfth hour, all colonies had cleaned 100% of cells containing dead brood.

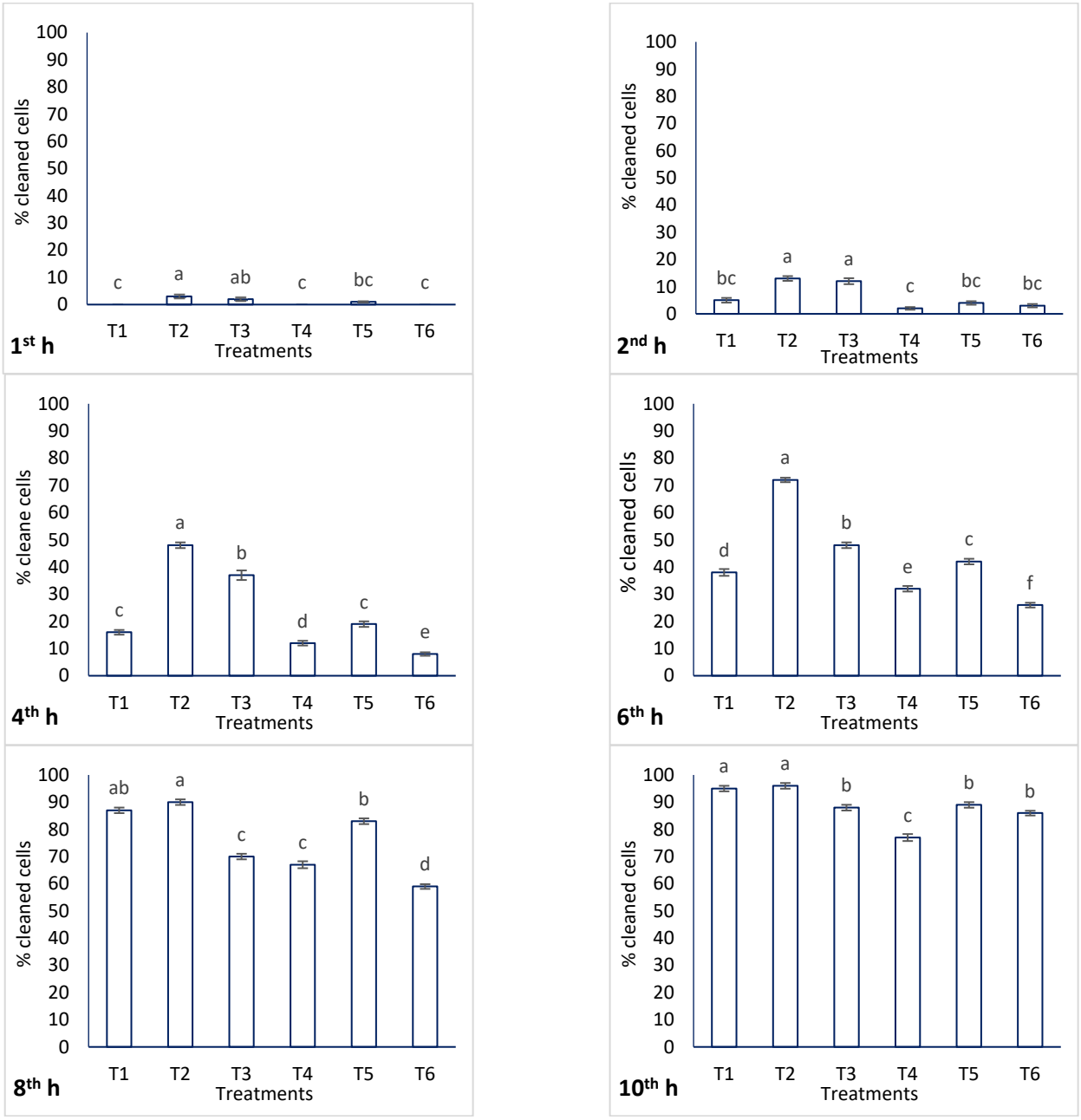


**Figure 3.** *Cont.*

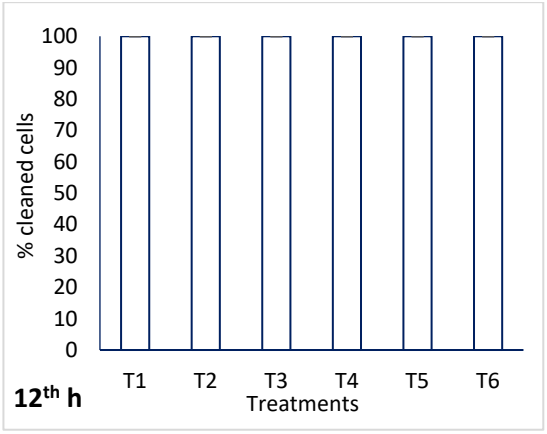


**Figure 3.** Percentage of cleaned cells in Yemeni and Carniolan honeybee colonies. Different letters indicate significant differences at the 0.05 level. $T_1$ = Yemeni colony and the pin-killed brood comb from the same colony, $T_2$ = Yemeni colony and the pin-killed brood comb from another colony of the Yemeni bees, $T_3$ = Yemeni colony and the pin-killed brood comb from a Carniolan bee colony, $T_4$ = Carniolan colony and the pin-killed brood comb from the same colony, $T_5$ = Carniolan colony and the pin-killed brood comb from another colony of the Carniolan bees, and $T_6$ = Carniolan colony and the pin-killed brood comb from a Yemeni bee colony.

Significant ($p < 0.001$) positive correlations (r = 0.63–0.80) were found between the number of workers that detected the dead brood cells and the percentage of uncapped and cleaned cells at 2, 4, 6, and 8 h after the brood were pin-killed in a comb, while the correlations were insignificant at the beginning of the experiment and 8 h after the pin-killed brood (Table 1).

**Table 1.** Pearson's correlation coefficients for the number of workers arrived at the dead brood cells and the % cleaned cells.

| Items | r | *p*-Value |
|---|---|---|
| W × C at 0 h | 0.29 | 0.1608 |
| W × C at 2nd hour | 0.80 ** | <0.0001 |
| W × C at 4th hour | 0.80 ** | <0.0001 |
| W × C at 6th hour | 0.65 ** | <0.0001 |
| W × C at 8th hour | 0.63 ** | <0.0009 |
| W × C at 10th hour | 0.06 | 0.7647 |

** Correlation is significant at the 0.01 level (2-tailed). C = % cleaned cells, W = number of workers arrived at the dead brood cells.

## 4. Discussion

The number of workers that detected the dead brood cells was different and significantly influenced by the race of workers who uncapped and cleaned the cells, the colony and race of the tested comb, and the interaction between these two factors. The differences in the number of workers that detected the dead brood cells due to these factors were significant ($p < 0.01$) at all inspection times, except for the effect of the race of workers in the first hour and the colony and race of the tested comb at the first and the second hour, which was significant at the 0.05 level (Table 1). The number of workers that detected the dead brood cells was variable and significantly depended on the race of the workers arrived at the cells, as well as the colony and race of the tested comb. Relatively similar results have been reported [1–3].

The hygienic behavior of a bee is identified based on the bee's ability to detect diseased or dead brood and uncapping and removing such brood from the comb cells. The detection

and removal of more than 90% of the diseased larvae from the comb cells have been defined as hygienic behavior [48]. In the current study, the Yemeni bee race showed a rapid response to detect the cells with dead brood compared to the Carniolan bee. More than 20.00 workers of Yemeni bee colonies ($T_1$, $T_2$, and $T_3$) arrived at the dead cells in the second hour after pin-killing the brood cells. The number of workers arriving at the dead cells in the fourth hour after pin-killing the brood cells exceeded 30.00 workers in the Yemeni bee ($T_1$, $T_2$, and $T_3$) and the Carniolan colonies and the pin-killed brood combs were from another colony of the Carniolan bees ($T_5$). The number of workers arriving at the dead cells was more than 40.00 and 60 workers in the sixth and eighth hour after pin-killing the brood cells, respectively, in the Yemeni bee ($T_1$, $T_2$, and $T_3$). In Carniolan bee colonies, the number of workers arriving at the dead cells was more than 50.00 and 60 workers in the eighth hour after pin-killing the brood cells in $T_4$ and $T_5$, respectively. The highest number of workers arrived at the dead cells in the eighth hour was 70.00 workers in $T_1$. After the eighth hour, the number of workers checking the pin-killing brood cells decreased due to the decline in the un-cleaned cells.

The behavior of removing the dead brood and cleaning the cells was different and significantly influenced by the race of workers who uncapped and cleaned the cells, the colony and race of the tested comb, and the interaction between these two factors. The differences in % of cleaned cells due to these factors were significant ($p < 0.01$) at all inspection times, except for the effect of the colony and race of the tested comb in the second hour, which was significant at the 0.05 level (Table 1). In this context, our results confirm the findings obtained by Kamel et al. [1] and Balhareth et al. [2], who found that the removal percentage of dead brood in Yemeni bee colonies was more than two-fold that of the Carniolan bees over time of inspection.

Some honeybee races can minimize the infestation level of the varroa mite by cutting the reproductive cycle of the mites in worker brood cells [22,49]. When workers in a colony uncap and remove more than 95% of the dead pupae within 48 h of killing that means they rapidly remove diseased or dead brood [17]. In the current study, the workers started to uncap and clean the cells containing dead brood in the first hour after pin-killing the brood cells in Yemeni and Carniolan bee colonies when the brood combs were of the same race but from different colonies ($T_2$ and $T_5$, respectively), and in Yemeni bee colonies when the brood combs were from the other race ($T_3$). The ratio of entirely cleaned cells increased gradually and reached 48.00% $T_2$ and 37.00% in $T_3$ at the fourth hour, while still less than 20.00% in the other treatments, and reached 72.00% in $T_2$ at the sixth hour after pin-killing the brood cells, and still less than 50.00% in the other treatments. At the eighth hour, the ratio of the cleaned cells reached 87.00, 90.00, 83.00, and 70.00% in $T_1$, $T_2$, $T_5$, and $T_3$ at the eighth hour, i.e., the % of entirely cleaned cells was $\geq$70.00% in all Yemeni colonies at the eighth hour after pin-killing the brood cells. At the tenth hour, these ratios reached 95.00, 96.00, 89.00, and 88.00%, respectively. All colonies cleaned 100.00% of the pin-killing brood cells after 12.00 h. These results confirm the findings obtained by Kamel et al. [1] and Balhareth et al. [2], who found that the removal percentage of dead brood in Yemeni bee colonies was more than two-fold that of the Carniolan bees over the time of inspection. In recent studies, Shakeel et al. [50] and Khan and Ghramh [3] showed that the removal percentage of the infested brood was significantly lower in the Carniolan (*A. mellifera carnica* Pollman) bee colonies in comparison with the Italian (*A. m. ligustica* Spinola) bee colonies, and this is may explain the lower mite infestation rate in Italian bee colonies in comparison with the Carniolan bee colonies. In addition, *A. m. carnica* bees have been shown to have a lower ability for dead brood removal in comparison with *A. m. mellifera* bees [51]. On the other hand, Carniolan bees have been shown to be slightly more hygienic than the Africanized honeybees [52].

Herein, the hygienic behavior was more effective in the colonies with small-cell-sized combs (Yemeni race) than those with wide-cell combs (Carniolan race). The rapid response to the removal of the dead brood of the Yemeni bee colonies may be related to the small size of the pupa in the cell, which is in fact related to cell comb size [53–56]. The higher

concentration of pheromones inside the small cells may have caused the more efficient removal of the dead brood [55]. This result confirms the findings of Olszewski et al. [11] and Dziechciarz et al. [42], who found that the dead brood removal was more efficient from small-cell combs than from standard-cell combs. The performance of the hygienic behavior of honeybee colonies with various genotypes is more dependent on the selection than the degree of polyandry. Hygienic behavior in colonies is due to the characteristics of individual components and can vary significantly depending on environmental conditions [57].

## 5. Conclusions

Based on the obtained results, under the environmental conditions of eastern Saudi Arabia, the Yemeni honeybees showed higher ability to detect and remove the dead brood from cell combs in comparison with the Carniolan honeybees. Furthermore, the removal of the dead brood from non-related individuals of the same race is more efficient than that from related bees or bees another race. The outstanding performance of a few individuals in the expression of various traits indicates their usefulness in carrying out breeding programs for Varroa resistance.

**Author Contributions:** Conceptualization, S.N.A.-K. and E.-K.A.T.; methodology, S.N.A.-K. and E.-K.A.T.; validation, S.N.A.-K. and E.-K.A.T.; formal analysis, S.N.A.-K. and E.-K.A.T.; investigation, E.-K.A.T. and S.N.A.-K.; resources, S.N.A.-K. and E.-K.A.T.; data curation, S.N.A.-K. and E.-K.A.T. writing—original draft preparation, S.N.A.-K. and E.-K.A.T.; writing—review and editing, S.N.A.-K. and E.-K.A.T.; visualization S.N.A.-K. and E.-K.A.T.; supervision, S.N.A.-K. and E.-K.A.T.; project administration, S.N.A.-K. and E.-K.A.T.; funding acquisition, S.N.A.-K. All authors have read and agreed to the published version of the manuscript.

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