



E-ISSN: 2320-7078

P-ISSN: 2349-6800

JEZS 2016; 4(5): 586-602

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Received: 23-07-2016

Accepted: 24-08-2016

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## Evaluation of the defensive behavior in two honeybee races Iranian honeybee (*Apis mellifera meda*) and Carniolan honeybee (*Apis mellifera carnica*) and grooming behavior of different bee races in controlling *Varroa destructor* mite in honey bee colonies in Iran

**Shakib Vaziritabar, Assadollah Aghamirkarimi and Sayed Mehdi Esmailzade**

### Abstract

Two honeybee races *Apis mellifera meda* (Indigenous) and *Apis mellifera carnica* (hybrid) were used to study and compare the evaluation of the defensive behavior and the grooming behavior of the two honeybee races was evaluated against *Varroa destructor* mite in honey bee colonies in Iran in 2015-2016. Results also indicated that the hygienic and grooming behavior of imported bee (*Apis mellifera carnica*) was more efficient as compared indigenous bee (*Apis mellifera meda*). The findings of the research revealed that the Carniolan honeybee hybrid colonies showed more effective defensive behavior against *Varroa destructor* (38.50%) as compared to indigenous (41.20%) honeybee colonies. The study also indicated that the number of covered combs with bees was lower in Iranian race (Non-aggressive and Aggressiveness), whereas the highest value was recorded with the aggressive Carniolan bees. Different forms of damage to the *Varroa destructor* mites may be due to the genetic origin of the bee race.

**Keywords:** Defensive behavior, *A. mellifera meda*, *A. mellifera carnica*, Grooming, *Varroa destructor*

### Introduction

Aggressive and swarming behaviors are the weak point of Iranian honeybee (*Apis mellifera meda*) in comparison the best honeybee races in the world and was well described by Tahmasbi *et al.*, 2014 [124]. Beekeeping is being practiced in Iran since long ago. Presently, the beekeepers rear both the indigenous bee (*Apis mellifera meda*) and Carniolan bee (*Apis mellifera carnica*). One of the major problems, facing beekeeping industry in Iran is the infestation of honeybee colonies with parasitic mite *Varroa destructor*. The initial observations have indicated that indigenous bee race (*Apis mellifera meda*) is less susceptible to *Varroa* mites as compared to the imported bee (*Apis mellifera carnica*) and this quality may be attributed to its more efficient defensive behavior. A worker bee can groom all the mites stuck to its body by its legs and jaws; otherwise, it does some special movements to attract other bees attention to make clean up the parasites from its body [91]. Colony defense is a social behavior that involves a complicated sequence of actions by many individual bees and a variety of traits ranging from aggressive (e.g., guarding, recruiting, alerting, attracting, culminating, biting, stinging, and pursuing) to docile or gentle expression [34, 65]. An elaborate defensive system has evolved in both open-nesting [66, 120] and cavity-dwelling honeybee species [96], in tandem with the attractiveness of colonies and their nests as food resources for predators [24, 65]. In several species, defensive behavior was found to be heritable [8] and related to fitness [36, 113]. A study by Spivak 1996 [116], found a relationship between hygienic behavior and the removal of mites from brood cells in the field. Test colonies with queens selected for hygienic behavior and fewer mites than the control colonies [109]. Hygienic behavior is the main mechanism by which workers detect the diseased brood, un-cap the cells and removes the infested larvae or pupae [110]. The parasitic mite *Varroa destructor* infests both the European honeybee (*Apis mellifera*) and the Asian honeybee (*Apis cerana*) which is also supposed as the original host of the parasite [67]. Low *Varroa* infestation in *Apis cerana* Indicates that the association between the host and the parasite during evolutionary processes

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Created certain resistance mechanisms including the actual removal of mite's infested workers brood and killing of phoretic *Varroa* by effective grooming behavior [91, 93]. A study by Buchler *et al.*, 1992 [17], that focused on compared grooming behavior in *Apis cerana* and *Apis mellifera* and found 75% successful mites removal in the case of *Apis cerana* and 48% in *Apis mellifera* by grooming behavior. *Apis cerana* is more effective in both removing mites and causing damage to mites than *Apis mellifera* [43]. A study by Zaitoun *et al.*, 2001 [146], determined the degree of damaged mites in *Apis mellifera syriaca* (the native bees in the area of study) and they found that the average percentage of injured mites was 22.8%. Aggression in honeybees is a sequence of behaviors triggered by the presence of a potential threat to the hive and initiated by the so-called guard bees, which form the first line of defense for a colony [1, 20, 21, 34, 57, 79]. During the first stage of aggression, bees show a typical alert posture, display increased locomotion, release of alarm pheromone and wing buzzing to recruit nest mates. During the second stage when a potential target is located, bees approach it flying or running, followed by the third stage, during which bees start to display threatening behavior towards the target, rapidly flying around it and contacting it. In the final stage, the attack culminates with bees actively biting and stinging the target. Alerted honeybees recruit nest mates for defense of the hive using an alarm pheromone, which is produced by glands of the sting apparatus [142]. Two behaviors of honey bees, hygienic and grooming, are mechanisms of defense against brood diseases and parasitic mites, including *Varroa destructor*. *Apis mellifera* colonies remove the worker brood infested with *Varroa destructor* mites from the nest (hygienic behavior), and groom the mites off other adult bees (grooming behavior). The complex nature of honeybee defensive behavior has stimulated the development of a variety of assays to measure aggression of different bee species or different colonies, and to determine the influence of genetic and environmental factors on aggressiveness [21, 29, 44, 49, 115]. Field assays utilize free-flying bees, measuring aggression of a hive by recording the number of stings left on an object waved in front of a hive entrance or above an open hive, along with the latency time between the initial disturbance and the first attack [48, 50, 52, 119, 136]. These assays are a powerful tool to decipher the sequence of events and the triggers inducing honeybee defensive behavior. One of the most important behaviors for the honey bee colony is reproduction. In honey bees, there are two levels of reproduction: the individual level (mating and oviposition) and the colony level (division or multiplication of the colony, generally known as swarming). Another well-known type of behavior in honey bees is colony defense consisting of recognition of predators, alerting nest mates and enacting anti-predator behavior [21, 34, 78]. Beekeepers have for a long time recognized these two behaviors, swarming and colony defense [31], and enacted breeding strategies to reduce their expression, in opposition to natural selection [75, 82, 102, 132]. For example, the natural way for honey bee colonies to reproduce is to swarm, and this behavior is thus intimately connected to fitness, but in contrast to this, beekeepers favor colonies that never swarm. Likewise, defensive behavior is not favored by beekeepers, but very docile honey bee colonies can easily fall prey to natural enemies, like wasps, birds or mammals. Hence, maintaining honey bees with optimal behavior from a beekeeping point of view, at the same time maintains the demand for continuous artificial selection, at least until fixation occurs, i.e. unfavorable traits are removed entirely from a population. Such fixation, however, has not been

achieved, which is a strong argument for the idea that honey bees should not be considered as "domesticated". More recently, behaviors related to colony health and disease control, such as hygienic behavior and grooming have gained more interest among selection programs [7, 11, 55, 100, 103, 116]. This type of artificial selection seems to support natural selection, as increasing hygienic or grooming behavior should help the bees to remove several pathogens and parasites otherwise causing diseases. However, the fact that bees show variability for the expression of such traits can suggest that either no strong fitness values are attached to them or that an optimum colony composition exists, which is based on the distribution of workers to various tasks [89, 101, 126]. Feral and domesticated honey bee colonies have evolved elaborate defense mechanisms to protect both themselves and their food from pathogen and parasite invasion. The defense mechanisms of individual bees serve to minimize the threat for the whole colony [11]. Constitutional defense mechanisms, such as the chitinous cuticle, which serves as a barrier between internal and external environment, and the intestinal micro flora of the bee gut, can protect each individual bee against infectious diseases [41, 53]. Cellular defense mechanisms (haemocytes) and humoral reactions (enzyme and anti-microbic factors) can contribute to resistance toward infections [28, 64, 80]. The proventricular valve enables the bees to filter ingested spores, which serves as a mechanism of physiological resistance to diseases [41]. These individual responses, coupled with the short life-span of the bees and their rapid replacement with healthy individuals, can limit the spread of infections between bees within colony [11]. Aggressive and swarming behaviors are the weak point of Iranian honeybee (*Apis mellifera meda*) in comparison to the best honeybee races in the world [125]. Behavioral defense of the honey bee *Apis mellifera* against ectoparasitic mite *Varroa destructor* [5], involves two important mechanisms: hygienic and grooming behaviors. Foremost, hygienic (removal) and grooming behavior was described as the main mechanism by which *A. mellifera* resist the brood diseases like American foulbrood and chalkbrood. Hygienic honey bee workers have the ability to detect diseased brood, uncap the wax covering over the brood cells and remove infected larvae or pupae. Afterwards, it has been demonstrated that hygienic bees detect and remove pupae infested with the parasitic *Varroa* mites [116]. Arathi *et al.* 2000 [4], found that hygienic behavior is predominantly performed by the middle-aged worker bees that have not yet begun foraging and that 18% of the bees in the colony are actually involved in the task at any given time. The removal of infested pupae interrupts their production of the fertile mites inside sealed brood cells. In addition, the immature mites are killed which decreases the average number of offspring per mother mite [47, 95, 107]. In grooming, adult bees detect and remove phoretic mites from themselves (auto-grooming) or from nest mates (allo-grooming) [91]. In the process, the legs of the mite may be cut off or the cuticle of the idiosoma may be damaged by the bees' mandibles, causing the damaged mite to fall to the bottom of the colony [97, 105]. A study by Lodesani *et al.* 1996 [68] and Rosenkranz *et al.* 1997 [99], have revealed that, the main type of damage to the mite caused by successful grooming is amputation or mutilation of one or more legs. Injuries to the mites' idiosoma or gnathosoma are relatively rare. The colonies of *A. mellifera* die from *Varroasis* (disease caused by *V. destructor*) within a few years if the mite population growth is not regulated by the beekeeper. Chemical control has its problems and limitations - reduced efficacy and development of resistance to chemical

control by *Varroa* mites [72, 137]. Therefore, the only possible solution to the problems of honey bee *Varroasis* is the identification and use of resistant stocks of honey bees, and their selection for enhanced resistance toward that disease, which could be acquired with stimulating hygienic and grooming behaviors, without losses of reproductive-productive features of honey bee colonies [92]. There is considerable variation in defensive behavior both between and within honeybee species [90, 141], even in colonies headed by related queens kept under identical conditions in the same apiary [32, 118, 121, 131]. Factors causing such variability in defensive behavioral phenotypes include social (non-linear) interactions [19, 58], location [83], weather conditions [21, 38, 119], colony size [29, 58], nectar flow [69], honey stores [143], electric charges [144], the endogenous circadian clock [125], season and time of day [140], inbreeding of queen and workers [23], maternal effects [22], quantitative trait loci and paternal dominance effects [48, 51], and subjectivity of the observer [24]. The docility of honeybee colonies is an important factor in practical beekeeping; consequently, this trait is an important selection trait in honeybee breeding [9, 10]. A variety of methods have been developed to evaluate defensive behavior in honeybee colonies, and assorted stimulation regimes have been used to measure behavior objectively [65, 121, 127, 140]. However, several behavioral parameters cannot be measured easily and are, therefore, usually graded into classes according to observer judgment [74, 123, 133, 138]. In breeding programs, honeybee defensive behavior is usually assessed subjectively [24, 29, 104], a technique common in behavioral studies of agricultural species [6, 37, 134, 135] mentioned that subjective methods must be provided or be generated in the initial phase of behavior evaluation experiments. However, one limitation of this method, apart from concerns about susceptibility to biases, is that it may not be equally effective for all breeds within the species [135]. Thus, each rating scale must be validated as it enters into common usage. The use of chemicals or natural products for controlling *Varroa* mites, the most dangerous parasite to honey bee colonies, is not quite enough. The options for controlling *Varroa* mites by using colony management or biotechnical measures alone are limited [40]. So the priority should be references to the field of genetic improvement of the natural defense mechanisms against *Varroa* mites [98, 112]. Some bee races could resist *Varroa* infestation without any treatment and this was mainly due to a high percentage of infertile female mites in worker brood cells. Several characters of honey bee can affect resistance / tolerance to *Varroa* mites as grooming and brood removal behaviors (hygienic behavior). Grooming is a possible factor in limiting mite success. Grooming could lower numbers of offspring [46]. Besides the grooming activity of the bees, the damage rate under field conditions is certainly influenced by several factors such as mite mortality inside the brood cells and other potential mite predators [13, 59]. The hygienic behavior, the primary natural defense against some diseases, is believed to be controlled by two independently assorting, recessive genes: one for uncapping and another for removing diseased brood from the nest. Mite population growth in individual colonies was negatively correlated with reduced infestation after 40h of brood exposure and with reduced mite fertility after one week [129]. The antennae sensilla organs of worker bees play an important role in the natural defense behaviors against *Varroa* mites. They responded to the stimuli from mite infested workers as well as brood cells. There is a relationship between the natural defense behaviors and number and sort of the sensilla organs in the honey bees

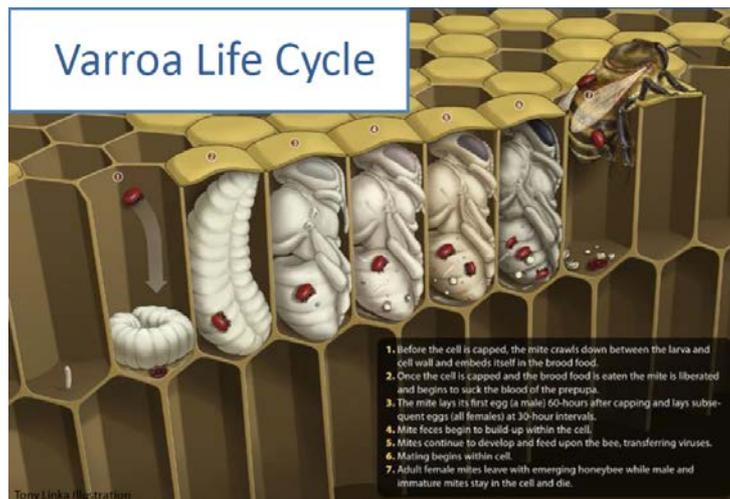
infested and non - infested with *Varroa* mites [108]. The parasitic mite, *Varroa destructor* Anderson and Trueman, previously named *Varroa jacobsoni* Oud, is an external parasite of honey bees which was reported for first time by Oudemans in 1904 from *Apis cerana* colonies in Java and then from Singapore in 1957. For the first time, in Hong Kong and Philippine in 1962-63, saw the *Varroa* mite in *Apis mellifera* L. colonies. Since then, the mite spread worldwide. In Asia, six *Varroa* haplotype that has selected *Apis cerana* as a host, named *Varroa destructor* [5]. Only two Japanese and Korean genotypes of *Varroa destructor* have been external parasites of *Apis mellifera*. *V. destructor* has been a serious threat to the Western honey bee *Apis mellifera* for almost three decades, nevertheless the classification of the virulent mite as a new species of *V. destructor* was recently made. *V. destructor* was known as *V. jacobsoni*, a species described from Java, which infest the Asian honey bee *A. cerana*. Other mites infesting *A. cerana* were similar to this species and were therefore classified as *V. jacobsoni* as well. A study by Anderson and Trueman 2000 [5] showed that *V. jacobsoni* is a species complex consisted from at least two species; *V. jacobsoni* and *V. destructor* both containing several haplotypes including the Korean haplotype and the less virulent Japan haplotype. *V. destructor* is only one species which has become a serious parasite of the Western bee *A. mellifera*. The original host of *V. destructor* is the Asian honey bee *A. cerana*. *V. destructor* was initially spread to *A. mellifera* by importation of *A. mellifera* to Asia.

### 1.1 Life cycle and biology of *Varroa destructor*

*Varroa destructor* can reproduce just in sealed brood cells of drone or worker bees. 15-20 hours before capping of the brood cells, the female *Varroa* leaves the adult bee body (which was its parasite) and transfer into brood, which are in their fifth instar period (Figure 1). While the young bee completes its metamorphosis period and leaves its cell, the female mite and its mature daughters leave the cell. These female mites transformed to other young bees easily. They live 4-13 days on mature bees before turning their parasitic stage to fifth instar larvae of bees. This phase of *Varroa* life cycle called as phoretic phase [62]. The success rate of reproduction (new mature female mites) in worker brood is about 1.7-2 but increases to between 2 and 3 in drone brood due to the longer development period [91]. *Varroa* are relatively large parasites compared to their host. *Varroa* are darkish red, reddish-brown oval bodies, approximately (1.6 x 1.1mm) with the body surface covered in fine hairs and eight legs located at the front of the body (Figure 2). *Varroa* have well developed chelicerae (jaws) that are used to puncture and attach themselves to the body of their host. Although *Varroa* mites do not have eyes, they detect honey bees by smell and movement. They move fast and can easily move from the body of one honey bee to another. *Varroa* are completely dependent on honey bee colonies for survival and reproduction. *Varroa* cannot live separately from honey bees for more than few days. *Varroa* feed on the haemolymph (blood) of adult honey bees after piercing their soft membranes located between segments of the exoskeleton, usually between their lower abdominal plates. To reproduce, the parent *Varroa* enter the brood cell of the honey bee right before the cell is capped (Figure 3). Once the cell is capped, the parent *Varroa* lays eggs and pierces the developing bee brood, leaving a wound open in it. The eggs hatch, the offspring mate with each other and feed on the developing bee brood along with the parent mite. The numbers of *Varroa*

offspring generated in a brood cell depends on the time the cell remains capped. This is why *Varroa* are able to produce more offspring in drone brood (2 to 2.5 offspring in 14 to 15 days capped) rather than in worker brood (1.5 offspring in 12

days capped). Thus, *Varroa* are adapted to seeking out drone brood. As the new adult bee emerges from the brood cell, the parent mite and the female mite offspring are released.



**Fig 1:** The reproductive cycle of *Varroa destructor* within the sealed honey bee worker brood cell, with the normal sequence of the sexes of mite offspring. A female mite enters the brood cell shortly before capping; approximately 3 days later the first male egg is laid followed by up to four female eggs.



**Fig 2:** Illustration of adult female mite. Dorsal (left) and Ventral (right) *Varroa destructor* mite. Photos by (Alejandro Ibacache, University of Pontificia Católica de Valparaíso).



**Fig 3:** Illustrations of *Varroa destructor* infested brood (left photo by Shakib Vaziritabar) and *Varroa destructor* and honeybee emerge from brood (right photo by Giles San Martin).

### 1.2 Resistance behaviors against *Varroa destructor*

A worker bee can groom all the mites stuck to its body by its legs and jaws; otherwise, it does some special movements to attract other bees attention to make clean up the parasites from its body [91]. Frequency of grooming behavior in *Apis mellifera* L. is less than *Apis cerana*. Several researches show

that parasites injured due to bee bites [94, 99]. A negative and meaningful correlation between number of parasites injured by bees and amount of infestation of the whole colony with *Varroa destructor* had reported [81]. Therefore, grooming behavior is expressed on the basis of the injured parasites; and it is considered an important parameter in selecting colonies

resistant against *Varroa*; however, frequency of this behavior in *Apis mellifera* L. is not as severe as that in *Apis cerana* [43]. Grooming behavior is one of important defensive mechanisms of honeybees against *Varroa* [116]. Regular and small pits at the back of *Varroa* mites related to growth period and parasite evolution; and should not be mistake with probable hurts by bees or other insect hunters [35]. In some race of honey bees, worker bees detect fertile and egg-laying parasites, and try to take the infested brood out of the hives, while they do not care those honey bee pupa that have been attacked by sterile mites. At first, this behavior of bees was called “Suppressed Mite Reproduction (SMR)”, but later on and due to result of several researches it was substituted by “*Varroa* Sensitive Hygiene (VSH)” [59]. *Varroa* sensitive hygienic behavior is a congenital trait and controls the considered parasite population. *A. mellifera* is far less successful in defense against *V. destructor*, nevertheless some mechanisms to decrease mite infestation are present. To understand better the mite and bee relationship, defense mechanisms in honey bees have been extensively studied and differences insusceptibility of species and races of honeybees to *V. destructor* have been found. Many efforts have been made to propagate lines of bees with more pronounced defense mechanisms which may permit beekeepers to cease or at least reduce chemical control, thereby lowering operating costs.

### 1.3 Honey bee societies

Honey bees are asocial insects living in perennial colonies with overlapping generations, cooperative brood care and a reproductive division of labor. The colony consists of three castes: female worker bees which can number between 15000-50000 depending on the time of year with a peak in the summer and a dearth in the winter; a few hundred male drones usually present in the spring; and one reproducing female queen bee (Figure 4). All three castes have four developmental life stages: egg, larva, pupa, and adult. All except for the adult stage occur in single hexagonal wax comb cells built by the bees within the nest. These immature stages (collectively referred to as brood) are immobile and completely dependent on the care of their sisters for their own survival. Due to the haplodiploid system, fertilized eggs become diploid females and unfertilized eggs become haploid male drones. Whether a fertilized egg will develop into a worker bee or a queen depends on the quality of the food they are fed by their sisters during larval development. Worker bees rarely reproduce and instead devote their lives to helping raise their sisters. While worker bees still have the possibility to lay unfertilized eggs, the pheromones of the queen inhibit this behavior within the colony. The queen lays all the eggs for the colony at a rate of 2000 per day on average. The queen mates once in her life with several drones and is able to store all the sperm in her spermatheca for her entire life of about three years. Workers and drones have a life span of about 6 weeks with the exception of overwintering worker bees that can survive up to 8 months in a winter cluster. Drones have only one purpose to mate with virgin queens. Upon copulation the drone will die while any drones that did not manage to mate will be expelled from the colony in the autumn. The colony growth and reproductive fitness depend entirely on their productive efforts of a single queen bee. However, swarming is a process where the colony divides into two or more new colonies and produces new queens (Figure 4). This event is thus considered a form of colony-level reproduction where the colony reproduces as a single unit. Although some reproductive fitness may be achieved

through the production of drones and swarming is fundamental for colony fitness [75]. In social insects a host population consists of a number of groups such as colonies, each containing a number of host individuals [115].



**Fig 4:** Queen bee (top left); Iranian worker bees *Apis mellifera meda* (Indigenous) with *Varroa* mites on their thoraxes (top middle); swarm of bees hanging in a tree branch (top right); Carniolan worker bees *Apis mellifera carnica* (imported) with *Varroa* mites on their thoraxes (bottom left); grooming and hygienic behavior by Carniolan honey bee race *Apis mellifera carnica* (bottom middle) and drone bee *Apis mellifera carnica* (imported) with *Varroa* mites on their thoraxes (bottom right). Photos by Sh. Vaziritabar, University of Varamin-Pishva in Iran.

### 1.4 Behavioral defense facilitating mite mortality

Honeybees have developed defense mechanisms against pathogens. Behavioral defense of bees against the mite including grooming and hygienic behavior is well researched. Both mechanisms increase the mortality of mites and thus decrease the colony infestation although not sufficiently for the colony to survive. Environmental conditions have also been suggested to play a role in the development of the *Varroa* mite population within a colony [39, 76], however it is more likely that this is only observed through the indirect effect of environmental factors that regulate honey bee brood amounts or the activeness of certain host defense behaviors. A well-known behavioral defense is grooming behavior, where honey bees groom themselves and other nest mates resulting in the capturing and damaging of adult mites [15, 73, 81, 91]. Hygienic behavior, another well-known behavioral defense of honey bees, is the ability to detect and remove dead or diseased brood [11, 103, 116]. A variation of hygienic behavior involves the ability to detect and remove mite infested brood and has been termed *Varroa*-sensitive hygienic behavior [59, 62, 116]. The removal of mite infested brood however does not necessarily include the death of the mite and most mites escape during the removal process [11]. Nevertheless, this behavior results in an interruption of the mite's reproductive cycle that ultimately could slow down the mite population growth in the colony. It is not well known how the bees are able to recognize the mite on nest mates during grooming behavior or within brood cells during hygienic behavior since the *Varroa* mite has a similar cuticular hydrocarbon profile to their host bee [84] used for chemical mimicry especially within the brood cells [71, 114]. It may be that mite infested brood is recognized by an unspecific stress reaction of the pupae [3]. Bee defense behaviors are highly variable between bee species and races [43, 70] and quantifying the trait accurately depends strongly on the methods used. Recently it has been discovered that damage to the dorsal surface of the mite, a characteristic that has been used to quantify grooming behavior, is actually a normal birth defect of mites [35].

Further, mutilated mites may have been damaged after they have died naturally or by other insects such as ants that scavenge on bottom boards in colony debris [99]. The invasion rates of the mite to brood of different origin are still largely unknown. Knowledge of the invasion rates for different races of bees would be valuable in simulations of population growth of *V. destructor* and thus could be included in the programs selecting bees for mite resistance.

### 1.5 Grooming behavior

Grooming behavior is the dominant natural defense against brood diseases of the honey bee such as American Foulbrood (*Paenibacillus larvae larvae*) and chalkbrood (*Ascosphaera apis*) reviewed in Spivak and Gilliam 1998a; b, and is also a natural defense against *V. destructor* mites infesting brood cells reviewed in [11]. As a behavioral trait of the bees, it might contribute to overall resistance against *V. destructor*. Honey bees with hygienic behavior detect, uncapped, and remove diseased, infested, or parasitized brood from the comb [18]. Grooming behavior allows bees to remove mites from their bodies and was well described by Peng *et al.* 1987 [91]. Grooming behavior includes self-grooming and nest-mate grooming performed by uninfected workers. The infested worker which could not remove the mite by itself performs the grooming dance by vibrating its body laterally. Workers triggered by the dance approach the bee, which stops dancing and stretches the wings and legs and rises up its thorax and abdomen. The nest-mates examine the body with antennae and remove the mite with the mandibles. In the process of removing mites from the bee's body, mites could be damaged [91]. Many authors have compared grooming behavior between the Asian bee, *A. cerana* and the Western bee, *A. mellifera*. Regardless of the differences in the proportion of mites removed by bees, all reports are conclusive that *A. cerana* showed more intensive grooming behavior than *A. mellifera* [17, 47, 91]. *A. cerana* is capable of removing almost all mites, compared to *A. mellifera* which removes only a small portion of mites [17, 91]. In addition, significantly more mites were injured by *A. cerana* than *A. mellifera* and consequently fewer mites recover [17, 43, 91]. There is no direct evidence that grooming under natural conditions in *A. cerana*, plays a significant role in resistance to *Varroa destructor* [14]. Pronounced grooming behavior after half an hour of inoculation with the *Varroa* mite was reported in Africanized bees, which removed 38% in the contrast to *A. mellifera* which removed only 5.7% [76]. A negative correlation between mite population growth and the number of injured mites found by Arechavaleta-Valasco and Guzman-Novoa 2001 [2] and Moosbeckhofer 1992 [81], suggests that grooming may play an important role in reducing population growth of *V. destructor*.

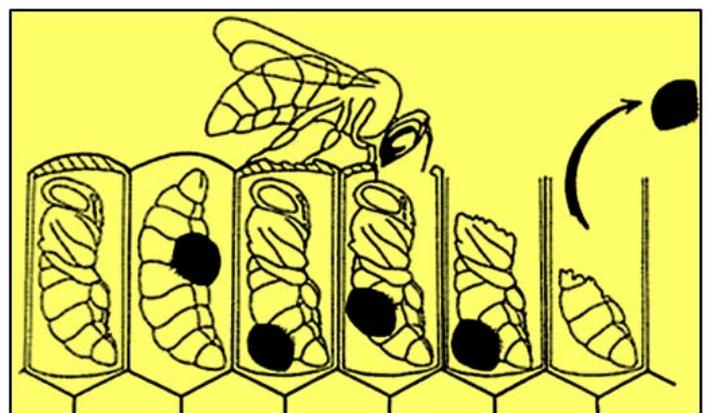
### 1.6 Hygienic behavior

Hygienic behavior is a mechanism of resistance to American foulbrood [111], chalk brood [55] and *V. destructor* [16]. The hygienic behavior of honey bees is a defensive mechanism including uncapping brood cells and removing diseased or dead brood. Hygienic behavior is genetically determined [77], however, [117] suggested it is also affected by colony strength and composition of workers within the colony. Hygienic behavior is tested by several methods including removing artificially infested brood with *V. destructor*, removing freeze killed brood or pin killed brood after some period. Workers removed double infested brood and freeze killed brood more frequently than single infested brood [16]. *A. cerana* is very effective in detecting and removing mites in worker brood [91,

95]. *Cerana* workers are not inclined to remove infested drone brood but in four days remove almost all mites (94%) in worker brood [95]. In contrast, *A. mellifera* in general showed lower removal response toward worker infested brood than *A. cerana*. A study by Boecking and Drescher 1992 [16], have revealed that, a removal rate of 24% in single infested cells and 41% removal response in double infested cells. However, there is high variability in removal responses between strains of *A. mellifera*, ranging from 5.5% to 96% in single infested cells and 5% to 100% in double infested worker cells [16]. A study by Janmaat and Winston 2000 [63], reported the influence of pollen storage on removal rate of workers infested with *V. destructor*. Low pollen colonies with a high demand for pollen performed high foraging that leads to a reduction in the number of bees engaged in cell removal behavior. *Varroa destructor* mite parasite alternates between feeding on blood of adult bees, and feeding and reproducing on the pupal stage of bees. Bees that remove mite-infested pupae from the nest interrupt the reproductive cycle of the mite by eliminating the offspring of the mite developing within a wax-sealed cell (Figures 5 and 6).

## 2. Materials and Methods

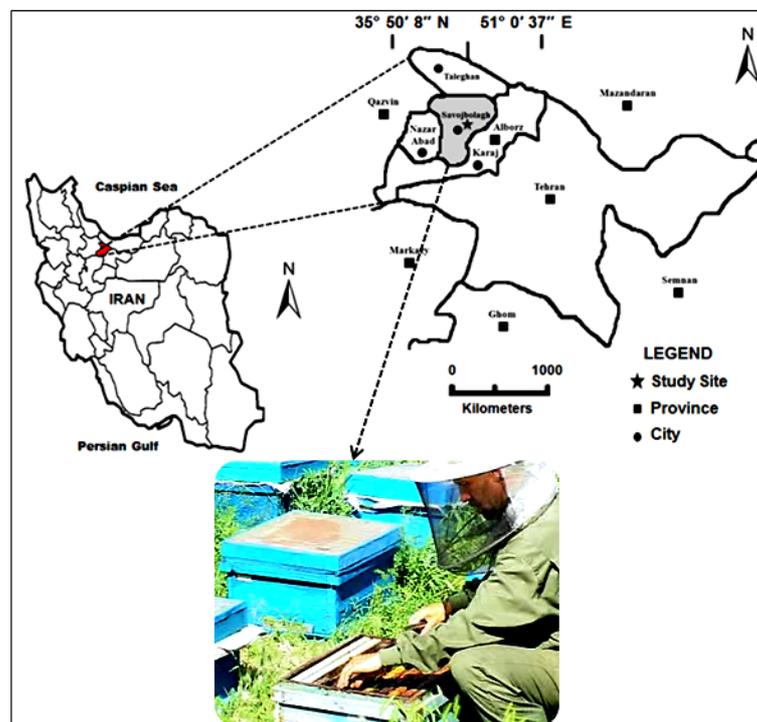
The aim of this study was to evaluate the defensive behavior in two honeybee races Iranian honey bee *Apis mellifera meda* (Indigenous) and Carniolan honey bee *Apis mellifera carnica* (imported) and grooming behavior of different bee races against *Varroa destructor* mite in honey bee colonies in Iran in 2016. The present study was conducted to investigate the grooming behavior of indigenous bee (*Apis mellifera meda*) and Carniolan bee (*Apis mellifera carnica*). The selection from the Iranian honey bee *Apis mellifera meda* (Indigenous) for the higher bee colonies purity and productivity was carried out in Savojbolagh region in Alborz province during a constant warm and dry weather in two years 2015-2016 (Figure 7). 10 colonies from two races (5 colonies from each race): *Apis mellifera meda* (Indigenous) and *Apis mellifera carnica* (Carniolan) were used. The colonies were similar in strength (7-combs covered with bees containing an average of 3-brood combs). The tested bee colonies did not receive any chemical treatments against *Varroa* mites during the experiment period.



**Fig 5:** Illustration of the process of removal, or hygienic behavior. A bee detects a cell containing a parasitized pupa. She uncaps the cell and the pupa is removed from the cell. The mother mite may escape from the cell, but her offspring would be destroyed by the bees during the removal process. (Drawing by O. Boecking).



**Fig 6:** Illustration of *Varroa destructor* sensitive hygiene (VSH). Workers uncaps cells to thwart reproduction by the mite. The beekeeper will observe exposed white and purple-eyed pupal faces. The pupa may or may not be chewed out in the process and one bee in the upper right quadrant is chewing out a pupa. Premature uncapping and chewing out of brood with mite in honey bee colonies in Iran. Photos by Sh.Vaziritabar, University of Varamin-Pishva in Iran.



**Fig 7:** Map of Iran and the study area of Savojbolagh apiary in Alborz province during (2015-2016).

## 2.1 Determine the nature of defense behaviors in the tested colonies

### 2.1.1 Grooming behavior

The analysis and evaluation of the honey bee grooming behavior were done according to the procedure of [60], whilst classification and quantification of the damage to *Varroa destructor* in accordance was done with the criteria established by Correa-Marques *et al.* 2000 [27]. Fallen dead and alive *Varroa* mites on a sheet of white paper placed on the bottom board of the bee colonies, were collected every 2 hours intervals/ 3 days /week between 10.am and 4.pm during the heavy infestation in warm and dry weather in two years 2015-2016 (Figure 8). The mites will naturally fall from the bees in the hive and be trapped in the adhesive material. A screen of 1/8 inch hardware cloth must be placed 1/4 inch above the sticky paper to prevent bees from becoming en-

tangled on the trap or allowing bees to remove mites from the trap. The collected mites were cleared by Nesbitts solution and mounted in Hoyer's medium and then the biting characters occurred on the body mites were detected and classified into 6 main symptoms representing biting or separating different part of the *Varroa* body;

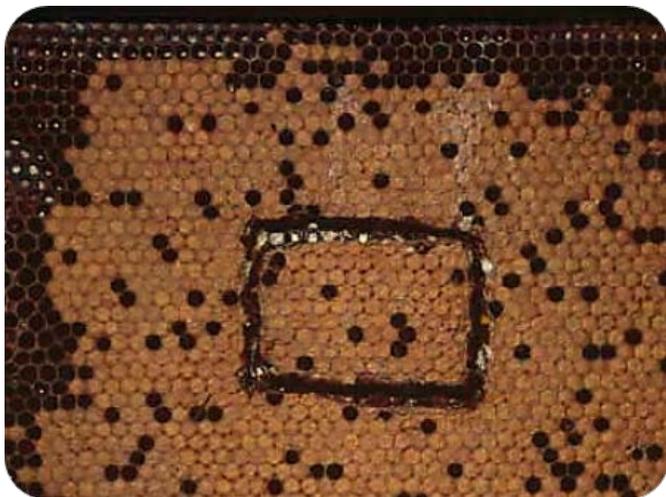
- 2.1.1.1 Gnathsoma and some segments of the legs.
- 2.1.1.2 Gnathsoma and all segments of legs except coxae.
- 2.1.1.3 Gnathsoma, some segments of the legs and all segments of legs except coxae.
- 2.1.1.4 Gnathsoma, legs from coxae and parts of the abdomen.
- 2.1.1.5 The dorsal shield.
- 2.1.1.6 Biting immature *Varroa* mites.



**Fig 8:** Photograph of Savojbolagh beekeepers use Iranian Longstroth wooden hive (top left illustration) A piece of sticky white construction paper is placed on the hive floor and screened floor suitable for monitoring natural mite fall (bottom left). *Varroa* floor with insert covered in hive debris (top right). Hive floor debris containing mites and mites knocked down onto sticky insert (circled in red) and a magnifying glass or dissecting microscope may be helpful in counting mites if heavy debris is present on the trap (bottom right). (Top left and bottom right Photos by Sh.Vaziritabar, University of Varamin-Pishva in Iran) and also (Top right and bottom left photos by The Animal and Plant Health Agency, National Agri-Food Innovation Campus, Sand Hutton York, YO411LZ) UK, Copyright in the typographical arrangement and design rests with the Crown copyright 2015.

### 2.1.2 Hygienic behavior (cleaning)

Sealed worker brood from each tested bee colony was divided into pieces of squares (2 inch square) and frozen for 48 h., then placed into brood combs of heavy infested bee colonies (Figure 9). The removal of dead brood cells by the worker bees were estimated at 24 and 48 hours for 3days/week/treatment respectively. Score for each colony; 1- Didn't clean. 2-Poor (cleaned in two weeks). 3- Good (cleaned in 48 h.) and 4- Excellent (Cleared in 24 h.).



**Fig 9:** A comb section of sealed brood containing approximately 100 cells on each side was cut out of the comb, frozen for at least 24 hours, and replaced in the hole left in the comb.

## 2.2 Experimental design

The *Varroa* mite is one of the most important parasites of honeybees that make heavy damage to the apiaries in the world. This mite also effect on honeybee behavior and cause colony collapse and reduce activity in hives. The purpose of this study was to investigate the effect of *Varroa destructor* mite on aggressive behavior in two honeybee races Iranian honey bee and European honeybee hybrid. For evaluating aggressiveness of bees, a modified technique of [42, 122] was

tested with a grayed on the black leather ball before swinging the ball in front of the entrance hole of the hive to provoke even the quiet colonies and black leather ball that was moved 50 cm in front of the hive entrance for half an hour between every two testes. For this purpose, infected and uninfected colonies were prepared and number of bees that who enter and leave the hive and also the number of sting that exist on black leather balls that move for half an hour between every two tests (Figures10 and 11) and Next the stings left in the ball were counted [140].

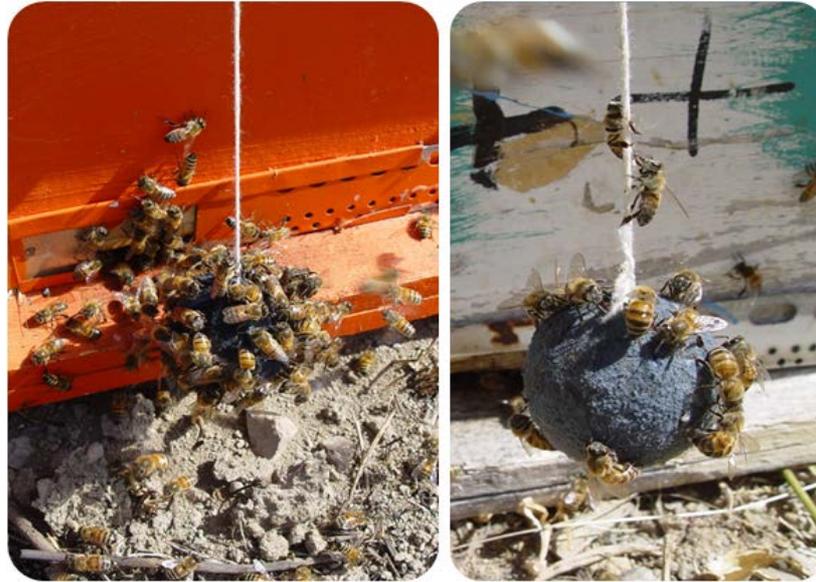
## 2.3 Investigating behavioral defenses

### 2.3.1 Evaluation of the aggressive behavior in two races colonies

We used a 'traditional' behavioral assay to test the stinging behavior of the experimental colonies (see Guzman- Novoa *et al.* 2003 [50] for more details about methods for testing the defensiveness of honeybee colonies). A modified technique of [42, 122] was used for evaluating aggressiveness of two honeybee races *Apis mellifera meda* (Indigenous) and Carniolan honey bee *Apis mellifera carnica* (imported). Next the stings left in the ball were counted [140] (Figure 11). Docility was recorded subjectively according to the Apimondia International Honeybee Breeding Recommendations [104], in which the scoring system during inspection is defined in terms of subjective criteria. The scores range from 1 (aggressive) to 4 (gentle). Intermediate marks (e.g., 2.5) are possible. Colonies were scored as Grade 1 when the observer was required to wear full protective clothing (veil, bee suit, and gloves) and when, despite continuous smoking during the inspection, the bees attacked continuously. Colonies were scored as Grade 2 when the observer was required to wear full protective clothing and smoking was required, but the bees made solo attacks without mounting a collective defense. Colonies were scored as Grade 3 when the observer was required to smoke but no protective gear was required. Colonies were scored as Grade 4 when no smoker or protective gear was required. Beekeepers in Iran always work fully protected (veil, bee suit, and gloves) at

their colonies whereas beekeepers in other countries (and with other bee races) usually dispense entirely with protective clothing. Consequently, this international scoring system is mainly based on a subjective assessment of what kind of protection/treatment is theoretically needed rather than what is practically used/carried out. To compensate for specific assessment time dependent environmental effects, all colonies in an apiary were tested during the same visit (one after the

other). Consequently, as in all honeybee breeding programs, we cannot exclude the possibility that the response of previously measured colonies might interfere with the evaluation of adjacent colonies to be measured later. Different assessment order colonies within the apiary was used to randomize this effect.



**Fig 10:** Illustrations are shown with black leather ball before swinging the ball in front of the entrance hole of the hive to provoke even the quiet colonies and black leather ball that was moved 50 cm in front of the hive entrance. (Photos by Sh.Vaziritabar, University of Varamin-Pishva in Iran).



**Fig 11:** Illustrations are shown the stings left in the black leather ball were counted in the laboratory. (Photos by Sh.Vaziritabar, University of Varamin-Pishva in Iran).

### 2.3.2 Hygienic behavior

The method used for determining hygienic behavior was chosen for its practicality in the field. One hundred pupae in each colony were marked and killed by piercing the pupal capping with a small insect pin [88]. One hundred cells in the same brood area were marked without being killed to serve as a within-colony control. The proportion of pupae that were removed 24 and 48 hours after pin killing was recorded. These proportions expressed the brood removal rate, or hygienic behavior of the colony.

### 2.3.3 Grooming behavior

Colony debris, including damaged mites, was collected using bottom board metal slide-in trays. The proportion of total mites found in colony debris that were damaged was recorded and used as a quantitative measure of adult bee grooming behavior [12]. The variations in grooming behavior during active season (April to October 2015-2016) were estimated by counting of damaged mites dropped on insert placed under brood nest. The biting characters occurred on the body mites were detected and classified into six forms of damage of the

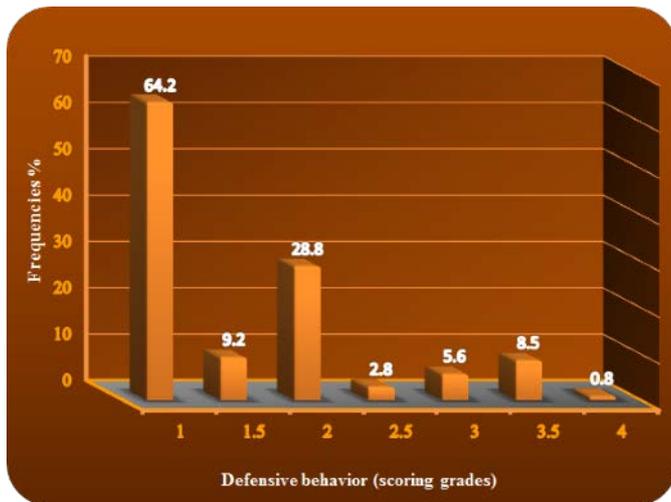
*Varroa* body.

## 2.4 Statistical model

SAS routines (SAS, 2008) were used to calculate frequencies and descriptive statistics of the defensive behavior.

## 3. Results and Discussion

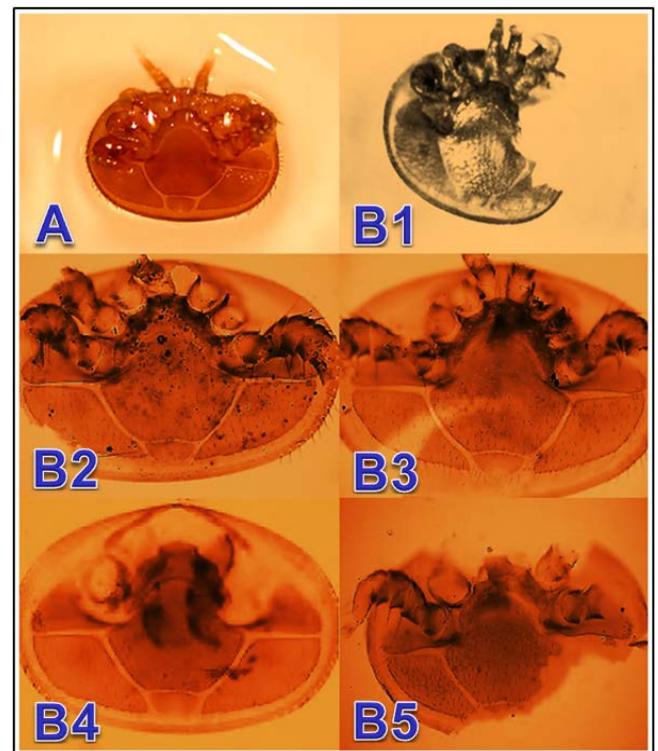
Using the scale of Ruttner 1972 [102], the average score for defensive behavior was  $1.35 \pm 0.42$ , which represents very aggressive colony behavior. In the tested two honeybee race colonies, 64.2% and 0.6% of total measurements were scored 1 and 4, respectively. Grade 4 was never given. A total of 91.6% of the total scoring grades were  $\leq 2$  (Figure 12). The standard deviation of individual scoring grades of the population was found to be 0.42. A total of 894 colonies were consistently scored as very aggressive during the whole observation period (Grade 1 only at all assessments). The findings of the research revealed that the indigenous bees race is less susceptible to *Varroa* mites as compared to the imported one and this quality may be attributed to its more efficient defensive behavior. The study also indicated that the number of covered combs with bees was lower in Iranian race (Non-aggressive and Aggressiveness), whereas the highest value was recorded with the aggressive Carniolan bees. Different forms of damage to the *Varroa destructor* mites may be due to the genetic origin of the bee race.



**Fig 12:** Defensive behavior was scored subjectively from 1 (aggressive) to 4 (gentle).

As shown in (Table 1) all the tested colonies (aggressive and non-aggressive) indicated the grooming behavior against *Varroa* mites. Also there were clear differences in this behavior according to the bee race and hybrid. The total Number of fallen *Varroa* mites due to the grooming behavior was higher in aggressive Carniolan hybrid race 333 mites (37.2%) followed with the Iranian race 158 mites (17.6%), and non-aggressive of Carniolan hybrid recorded the higher values 282 mites = 31.5% and Iranian recorded the lowest values of the fallen dead *Varroa* mites 121 mites = 13.5%, respectively. The microscopic studies of the damaged *Varroa* fallen due to the Grooming behavior, under laboratory condition in Iran showed several forms of damage. (Figure

13) as follows: A- Gnathosoma and segments of the legs. B- Gnathosoma and portions of the legs from coxae. C- Gnathosoma and portion of legs as segment and from coxae. D- Gnathosoma, legs from coxae and parts of the abdomen. E- The dorsal shield and F- Biting immature *Varroa* mites. The results also indicated that the damage to *Varroa* bodies represented the highest percentage in the class (A) 74.9%, from the total 99.8%, while the lowest was 13.5% and 17.6% for non-aggressive and aggressive Iranian race (indigenous), respectively. Aggressive Carniolan hybrid race (imported) recorded the highest percentage for the class (A) (74.9%), while the lowest percentage in the class (F) was with aggressive Carniolan hybrid race (4.2%). The class (B) showed higher percentage (25.3%) with the aggressive Iranian race, whereas the lower registration for the class (E) (13.8%) was in non-aggressive Iranian race (indigenous). The Iranian race (indigenous) distinguished by decisive percentage of the class (B) (46.9%) among other tested bee races and hybrids. Biting the dorsal shield (adj. E) showed lowest percentage in and between all classes of the cutting *Varroa* body.



**Fig 13:** Different kinds of damage to *Varroa destructor* due to the grooming behavior of honey bees. A- Normal outer morphology of *Varroa destructor* (ventral view); B- Different kinds of damage to *Varroa destructor* (1-5): B1- lack of two pair of legs reciprocal + damaged shields; B2- Biting of Gnathosoma and some segments of the leg and all segments of legs except coxae; B3- Biting of Gnathosoma and some segments of the legs; B4- Biting of Gnathosoma and legs from coxae and parts of abdomen; B5- Biting of Gnathosoma and some segments of the leg and all segments of legs except coxae. Photos [B2, 3, 4, 5] by Zakaria *et al.* 2009 and A photo by Alejandro Ibacache, University of Pontificia Católica de Valparaíso).

**Table 1:** Grooming behavior between two different Iranian bee race *Apis mellifera meda* (Indigenous) and Carniolan honey bee hybrid (imported) against *Varroa destructor* in honeybee colonies.

Bee race	Sampling Data	GB*	Adjectives of the grooming behavior						
			A	B	C	D	E	F	
Iranian race ( <i>Apis mellifera meda</i> )	Non - Aggressiveness	2 April	0	-	-	-	-	-	-
		9 April	12	-	-	-	-	-	-
		16 April	10	-	-	-	-	-	-
		23 April	15	-	-	-	-	-	-
		30 April	28	2 (7.1%)	2(7.1%)	3(10.7%)	1(3.5%)	1(3.5%)	-
		6 May	32	1(3.1%)	2(6.2%)	3(9.3%)	2(6.2%)	2(6.2%)	-
		13 May	24	2(8.3%)	2(8.3%)	2(8.3%)	2(8.3%)	1(4.1%)	-
	Total 121(13.5%)								
	Aggressiveness	20 May	0	-	-	-	-	-	-
		27 May	10	-	-	-	-	-	-
		3 June	28	2(7.1%)	2(7.1%)	1(3.5%)	1(3.5%)	2(7.1%)	-
		10 June	30	2(6.6%)	2(6.6%)	3(10%)	2(6.6%)	-	-
		17 June	14	-	-	-	-	-	-
		31 June	50	2(4%)	2(4%)	1(2%)	3(6%)	2(4%)	-
7 July		26	1(3.8%)	2(7.6%)	1(3.8%)	1(3.8%)	1(3.8%)	-	
Total 158 (17.6%)									
Carniolan hybrid race ( <i>Apis mellifera carnica</i> )	Non - Aggressiveness	14 July	44	2(4.5%)	3(6.8%)	2(4.5%)	2(4.5%)	2(4.5%)	1(2.2%)
		21 July	30	-	-	-	-	-	-
		28 July	10	-	-	-	-	-	-
		4 Aug	76	2(2.6%)	2(2.6%)	2(2.6%)	1(1.3%)	1(1.3%)	2 (2.6%)
		11 Aug	54	4(7.4%)	1(1.8%)	2(3.7%)	2(3.7%)	3(5.5%)	1(1.8%)
		18 Aug	28	-	-	-	-	-	-
		25 Aug.	40	2(5%)	2(5%)	1(2.5%)	3(7.5%)	2(5%)	1(2.5%)
	Total 282(31.5%)								
	Aggressiveness	1 Sep.	57	6(10.5%)	2(3.5%)	1(1.7%)	1(1.7%)	2(3.5%)	1(1.7%)
		8 Sep..	82	12(14.6%)	3(3.6%)	1(1.2%)	2(2.4%)	2(2.4%)	-
		15 Sep.	43	2(4.6%)	2(4.6%)	3(6.9%)	1(2.3%)	1(2.3%)	-
		22 Sep.	39	4(10.2%)	3(7.6%)	2(5.1%)	2(5.1%)	1(2.5%)	1(2.5%)
		29 Sep.	66	10(15.5%)	2(3%)	1(1.5%)	2(3%)	1(1.5%)	-
		5 Oct.	46	9(19.5%)	8(17%)	2(4.3%)	1(2.1%)	1(2.1%)	-
Total 333(37.2%)									
Total 894									

**A-**Cutting Gnathosoma and some segments of the legs. **B-** Cutting Gnathosoma and all segments of legs except coxae. **C-** Cutting Gnathosoma, some segments of the legs and all segments of legs except coxae. **D-** Cutting Gnathosoma, legs from coxae and parts of the abdomen. **E-** Cutting the dorsal shield. **F-** Biting immature *Varroa* mites. GB\* = (grooming behavior; fallen mites).

### 3.1 The hygienic behavior

The European bee hybrid race showed that the highest hygienic behavior of removal of dead frozen *Varroa destructor* mites during the first twenty four hour and 30% was faster than other tested bee races (Table 2). The grooming and hygienic behaviors in honey bee colonies may not be related to the aggressive behaviors of tested colonies. The tested colonies showed different forms of damage to the *Varroa destructor* mites; this may be due to the genetic origin of the bee race. In other words, the high honeybee frequency

in hive is not an indicator for intensity of defense behavior. Hence, some genetic studies have showed that the defense behavior has a high heritability, so it is expected being affected the genome of honeybee. Therefore it could be recommended to start an improvement hereditary program for naturally controlling *Varroa destructor* mite. Results presented in (Table 2) showed that the number of covered combs with bees was lower in Iranian race (Non-aggressive and Aggressiveness), whereas the highest value was recorded with the aggressive Carniolan bees.

**Table 2:** The hygienic behavior and biological activities of two honeybee races Iranian honeybee (*Apis mellifera meda*) and Carniolan hybrid honeybee (*Apis mellifera carnica*).

No.	Bee race	Hygienic behavior (%)	Sealed worker brood area (inch)	No. of covered combs with bees
1	Iranian race (Non-aggressive)	20	117	15
	Iranian race (Aggressiveness)	15	220	18
2	Carniolan hybrid(Non-aggressive)	30	250	19
	Carniolan hybrid (Aggressiveness)	15	100	22

Source: field survey, 2015.

Results presented in (Table 3) showed the total number of collected mites from two races of honeybee colonies and the percentage of damaged mites. A total 337 mites were collected from indigenous bee colonies (*Apis mellifera meda*) during the tested period. 58.80% of the total mites were found healthy while 41.20% were subjected to different type of damage due to the grooming activity. Similarly, a total of 350 mites were collected from Carniolan honeybee hybrid colonies (*Apis mellifera carnica*) as naturally dropped mites throughout the tested period. 61.50% of the total mites were found healthy where as 38.50% were subjected to different kinds of damage to *Varroa destructor* due to the grooming behavior of honey bees (Figures13). Kinds of damage were identified on legs, dorsal and appendage as 18.49, 14.49 and 8.38% respectively.

*Varroa* Sensitive Hygiene (VSH) is very similar or the same as hygienic behavior that honey bees use to combat American

foulbrood, chalkbrood, and the eggs and larvae of wax moths and small hive beetles. All colonies probably have individuals that perform VSH, and we do not yet understand how our selective breeding has resulted in colonies with greatly improved performance. Hygiene is performed by nest cleaning bees aged 15-18 days old. Removal of a mite-

infested pupae begins when an un-capper smells the infested brood and chews a pinhole through the cell cap was well described by Jeff Harris and Bob Danka 2010 [56]. Subsequently, removers enlarge the hole and either eat the infested pupa or pull it from the brood cell (Figures 14).



**Fig 14:** Premature uncapping and chewing out of brood with mite in honey bee hybrid colonies (*Apis mellifera carnica*) and mite biter in a survivor colony in Iran. Hygienic removal of a mite infested worker pupa by adult worker honeybee hybrid colonies (*Apis mellifera carnica*) is an important mechanism of resistance to *Varroa destructor* mites. The removal involves several bees, and results in death of the mite offspring. (Photo by Jeff Harris and Bob Danka: USDA-ARS, Baton Rouge, Louisiana. American Bee Research Conference. Orlando, Fl. January 15th, 2010).

**Table 3:** Total number of collected *Varroa destructor* mites from two races of honeybee colonies and percentage of damage

No.	Bee race	Total number of mites collected (mean)	Average of <i>Varroa destructor</i> mites (%)				
			Healthy mites	Damaged mites	Kind of damaged (%)		
					Legs	dorsal	App.
1	Iranian race (Indigenous)	337 (1.56)	58.80	41.20	15.41b	12.20d	14.08c
2	Carniolan hybrid (imported)	350 (2.06)	61.50	38.50	18.49a	14.49bc	8.38e

Source: field survey, 2015.

Results indicated in Table 3, showed that the Carniolan honeybee hybrid colonies (*Apis mellifera carnica*) showed more effective defensive behavior against *Varroa destructor* (38.50%) as compared to (41.20%) indigenous honeybee colonies (*Apis mellifera meda*). The mean of the collected mites was 1.56 (mites/colony/week) for indigenous bee races (*Apis mellifera meda*) while it was 2.06 mites for Carniolan honeybee hybrid race (*Apis mellifera carnica*).

Generally, behavior assessment depends on human perception and interpretation to varying degrees. Performance testing in the honeybee, including the assessments of defensive behavior, is conducted during the whole season. Thus, its influenced by multiple, uncontrolled climatic effects and suffers from potential confounding socio-environmental variables, such as colony size, endogenous circadian clocks, foraging activity, worker age, and other non-linear group interactions (e.g., release of or sensitivity to alarm pheromones, visual cues, or other social signals), which may all influence honeybees' defense behavior. Additionally, multiple observers will engage in a range of subjective evaluations, and individual observers also vary within a range for subjective evaluations across time. All these factors are likely to differ at each scoring time, thereby reducing the repeatability estimate. In general, it should be noted that repeatability estimates differ greatly according to the nature of the characteristics [8] and to the genetic properties of the population and the environmental conditions under which the individuals are kept [45, 128]. The number of bees that enter and exit the hive in healthy colonies is  $15.40 \pm 2.51$  and  $15.3 \pm 3.46$

and in infected colonies is  $8.25 \pm 1.55$  and  $8.38 \pm 1.53$  for 30 minutes. Also the number of sting on leather ball in healthy colonies is  $6.4 \pm 1.2$  and in infected colonies is  $15.6 \pm 1.95$  for 30 minutes. There is significant difference between aggressive and non-aggressive behavior in infected and healthy colonies. According to the obtained result it can be concluded that the *Varroa* mite infestation reduce foraging behavior and increase aggressive behavior and this phenomenon finally effects on hive power and cause colony collapse.

Hygienic and grooming behavior of the bees in resistant colonies against *Varroa* mites showed that hygienic and grooming behavior ability depends on relative number of these bees in the colony [68]. In a hygiene and grooming colony, relative number of those bees that do hygienic and grooming activities is just a few, but these bees do discovery activities, uncapping and removing infested brood very well [88]. In a study, percent of mites that infest the bee's brood and number of the mites that infest the adult bees (mites in phoretic stage) considered as a symbol of hygienic and grooming behavior; and while they showed that both These characteristics play a meaningful role, they estimated regression coefficient of brood infesting mites more than phoretic ones [61]. The development of the scoring system used, proposed by Ruttner (1972) [102], was strongly influenced by the behavior of *Apis mellifera carnica*, one of the most gentle bee subspecies. These Apimondia International Honeybee Breeding Recommendations are still widely used [25] and efficient for the comparison of different subspecies, but their rough scale may not sufficiently

discriminate differences in defensive behavior between colonies of *Apis mellifera meda*. Generally, behavior assessment depends on human perception and interpretation to varying degrees. Performance testing in the honeybee, including the assessments of defensive behavior, is conducted during the whole season. Thus, it is influenced by multiple, uncontrolled climatic effects and suffers from potential confounding socio-environmental variables, such as colony size, endogenous circadian clocks, foraging activity, worker age, and other non-linear group interactions (e.g., release of or sensitivity to alarm pheromones, visual cues, or other social signals), which may all influence honeybees' defense behavior. The number of grades within a scale has a positive relationship with its precision and sensitivity<sup>[130]</sup>. Broadening the scoring system to encompass a wider range of scores and/or including intermediate options has worked for other species<sup>[85, 87]</sup>. However,<sup>[6]</sup> mentioned that applying the same test on different populations for improving temperament may not reveal enough genetic variability to discriminate animals based on temperament. Therefore, in a uniform population, the rough scoring system is not only likely to lower the repeatability estimate but also to fail to efficiently detect the variation within a population. Even for *Apis mellifera carnica*, which has been selected for gentleness for several decades, this rating system may reach its limits. Using the scoring system, the average defensive behavior in an Austrian Carnica population was found to be  $3.12 \pm 0.46$ <sup>[139]</sup>, and it was  $3.73 \pm 0.63$  in a German Carnica population<sup>[22]</sup>, both with a right-skewed distribution. The scoring system is well suited to provide average insight into absolute differences between different races, but it is failing to detect efficiently the variation within a population having a certain degree of uniformity. Guzman- Novoa *et al.* 2003, scored the (1 – 5) defensive behavior of European and Africanized honeybee colonies relative to the scores given to the first colony tested. They found this rating system to be the most reliable, economical, and practical to separate gentle from defensive bee types. A study by Willam and Ebi 1993<sup>[139]</sup>, and Zakour *et al.* 2012<sup>[145]</sup>, reported quite small and identical heritability (0.08) for defensive behavior in a selected *Apis mellifera carnica* population and in an unselected Syrian honeybee population, respectively.

#### 4. Conclusion

Using a 'traditional' behavioral assay to investigate aggressiveness and stinging behavior of the experimental colonies, we have shown that total 337 mites were collected from indigenous bee colonies (*Apis mellifera meda*) during the tested period. 58.80% of the total mites were found healthy while 41.20% were subjected to different type of damage due to the grooming activity. Similarly, a total of 350 mites were collected from Carniolan honeybee hybrid colonies (*Apis mellifera carnica*) as naturally dropped mites throughout the tested period. 61.50% of the total mites were found healthy where as 38.50% were subjected to different kinds of damage to *Varroa destructor* due to the grooming behavior of honey bees. Kinds of damage were identified on legs, dorsal and appendage as 18.49, 14.49 and 8.38% respectively. Our results show that aggressive tested Carniolan hybrid honey bee is more aggressive than indigenous bee colonies (*Apis mellifera meda*). Carniolan honeybee hybrid colonies (*Apis mellifera carnica*) showed more effective defensive behavior against *Varroa destructor* (38.50%) as compared to (41.20%) indigenous honeybee colonies (*Apis mellifera meda*). Our results also show a

significant interaction between genotype and location, since colonies of local origin were considerably less defensive than introduced ones. This could indicate that the non-local genotypes expressed stronger defensive reactions due to the lack of adaptation with environmental conditions, such as higher or lower temperatures than in their local range, or the presence of different predators Arechavaleta-Velasco and Hunt, 2003<sup>[1]</sup>; Breed *et al.* 2004<sup>[21]</sup>. In conclusion, many factors seem to regulate the expression of hygienic behavior of honey bees and the removal of pupae infested with *Varroa*. Hygienic behavior has the potential to limit the population growth of *Varroa* in three ways: 1) the immature mites are killed when the pupa is removed, which decreases the average number of offspring per reproducing mite; 2) the phoretic period of adult female mites is extended of those mites that survive removal of the pupae; and 3) the mortality of the adult mites increases if they are damaged by the adult bees through grooming when they escape through the opened cell. These results indicate success of future programming for breeding Iranian honeybees in terms of resistance characteristics against diseases and *Varroa destructor* mite. In addition, results of our research show that Iranian honey bee *Apis mellifera meda* (Indigenous) has potential of resistance against *Varroa* mites without any drugs and chemicals; and one-can produce resistant bees for beekeepers through selection and breeding. Our data which show that the most frequent kind of damage is damaged legs are in accordance with those of<sup>[26, 68, 94, 99]</sup>.

#### 5. Acknowledgement

We thank all the beekeepers who contributed to this study and also for providing the hives infested with parasitic mite *Varroa destructor* mites. I also thank the beekeepers who donated their bee colonies to be used for the experiments. I would like to express my gratitude to A. Aghamirkarimi for providing helpful comments to carry out this research project. I wish to thank Mr. S.M. Esmaeilzade, for assisted with data collection and endless help during field work and the three anonymous reviewers for the improvement of the manuscript. We would like to thank Department of Entomology Research (honeybee laboratory) and Department of Animal Science Research Varamin - Pishva University for their cooperation and excellent facilitation during study period and we are also grateful to two anonymous reviewers for their insightful comments.

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