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Identification of differential defense reaction and tactic of Iranian honeybee *Apis mellifera meda* colonies under attack hornets *Vespa orientalis* and *Vespa crabro* in Iran

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Abstract

The aim of this study was to describe how Iranian honey bees *Apis mellifera meda* are able to defend their colonies against skirmish two hornets *Vespa orientalis* and *Vespa crabro* in Iran; Identification differential reaction and defensive tactics that operate at the colony level during predation, and are Iranian honey bees *Apis mellifera meda* able to kill the hornets and effective struggle or retreat under attack hornets? The present study analyzed hornet predatory behavior and collective response of colonies under attack. The results showed that Iranian honey bee *A. mellifera meda* in Iran exhibit an inefficient and unorganized defense against European hornet *Vespa crabro*. In some colonies, when attacked, the numbers of guards at the hive entrance increases rapidly to attack, engulf, and kill invading hornets. In other colonies, guards avoid conflicts with hornets by retreating gradually and by forming a defensive line of honeybees at the hive entrance.

Keywords: *Apis mellifera meda*, defense reaction, *Vespa orientalis*, *Vespa crabro*, skirmish

1. Introduction

Vespid hornets are important predators of insects including honey bees. Colony defense of Asian *Apis* species is characterized by several distinct behavioral elements, which are absent in the western honey bee *Apis mellifera* [2]. Guarding behavior is influenced by the environment [13, 17]. Downs and Ratnieks [23] found that guarding behavior, measured by number of guards and by the number of fights observed at the entrance of the hive, changes during a period of time depending on the robbing pressure from other colonies as a consequence of the presence or absence of nectar in the field. Honeybee (*Apis mellifera* L) defensive behavior is a complex trait that involves individual worker behavior and a coordinated colony response with a group effect [1]. Unfortunately, it is so far no studies exist regarding the defense behavior of Iranian honeybee *A. m. meda* against attack hornets *V. orientalis* and *V. crabro* (European hornet) in Iran. Many studies have described several coordinated and massive defense tactics performed by the honeybee colonies when attacked by hornets [39, 49, 63]. Regardless of the specific factors that cause hornet death during honeybees' defense, it appears that the key element that determines bees' successful defense against hornets is whether defenders are able to form a ball to fully engulf the predator. Examples include colony aggregation on the beehive platform in a manner called "bee-carpet behavior" Baracchi *et al.* [10] and Papachristoforou *et al.* [51], synchronized abdominal shaking known as shimmering Butler [16] and Kastberger *et al.* [38], emitted an alarm sound defined as hissing Papachristoforou *et al.* [50], and building walls of propolis to prevent hornets from getting into the hive Papachristoforou *et al.* [51]. Many species of hornets (Vespinæ) are serious enemies of honeybees [8, 45]. Most bees of the genus *Apis* can efficiently defend their nests when confronted by predators such as hornets or wasps [10, 16, 40, 49, 50, 55, 62, 69]. Ultimately, hornets can seriously damage colonies either by preying on honeybees or by robbing their resources (i.e. honey, pollen, and brood). These resources are then used to sustain their own brood and adults within the colony; a colony that in turn may go on to destroy the entire honeybee colony [8, 46]. The species composition and seasonal pattern of *Vespa* hornets were monitored from diverse environmental habitats in Iran area. Only two species, *V. crabro* and *V. orientalis*, naturally reached the European and Middle East areas, Carpenter and Kojima [19]. Scattered literature has presented data on the vespine wasps of Iran. Both species of hornets are present in Iran: *V.*

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orientalis Linnaeus, 1771, has a widespread distribution in most parts of Iran, except for the Caspian coast in northern Iran, but *V. crabro* Linnaeus, 1758, is present only on the Caspian coast, Ebrahimi and Carpenter [24]. *V. orientalis* is known as a common wasp in Iran. Esmaili and Rastegar [25], have reported both species. Ebrahimi and Carpenter [24] published new data on *Vespa* spp. in Iran and summarized their distribution. The objective of this study is to better understand how *A. m. meda* colonies (Iranian honeybee) defends its colonies against two predator, *V. orientalis* and *V. crabro*, by addressing the following questions: how do the two species, *V. orientalis* and *V. crabro*, interact at the entrance of the *A. m. meda* colony? Can Iranian honeybees develop an effective balling behavior against two predators? Are Iranian honeybees able to kill the hornet, and whether *A. m. meda* can effective struggle against attack hornets or retreat under attack hornets?

2. Materials and Methods

2.1 Experimental apiaries

This study was conducted for identification differential of defense reaction and tactic of *A. m. meda* colonies under attack hornets *V. orientalis* and *Vespa crabro* during (May 2016 - December 2017) in the Savojbolagh district, located between (35° 50' 8" N and 51° 0' 37" E) in the Alborz province of Iran (Fig. 1). We studied two apiaries in Savojbolagh in the Alborz province of Iran (site A and site B), consisting of 50 and 71 *A. m. meda* colonies respectively.

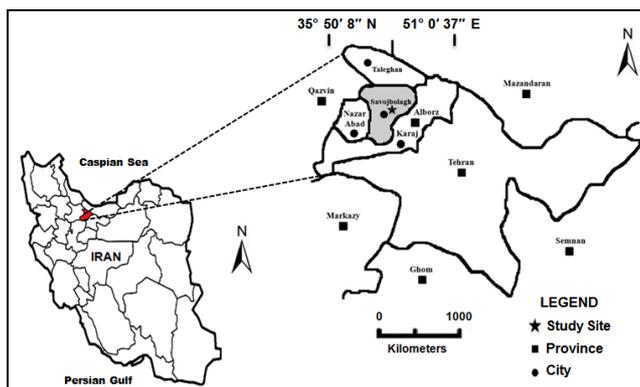


Fig 1: Map of Iran and two apiaries in Savojbolagh in the Alborz province site (A) and (B).

2.2 Behavioral observations

Agonistic events between hornets *Vespa orientalis* and *Vespa crabro* (European hornet) and *A. mellifera meda* (Iranian honey bee) were examined in two different contexts, one at the hive entrance (which was regularly patrolled by guard bees) and the other on the ground close to the hive, where weakened and dead bees were present. The behavioral observations were based on the “all occurrences sampling” method [3] in which we recorded the frequencies of a series of behavioral events as set out in the ethogram described below. Attacks at the nest entrance were recorded in 2016 and 2017 throughout January and October, when the predatory activity of wasps is more intense due to their higher nutritional requirements during reproduction and rearing of offspring [69]. Each colony was recorded for two 15 minute sessions per day using a Canon LEGRIA HF R506 video camera placed 50 cm from the hive’s flight board. This work took place in the middle of each month between May 2016 and December 2017 at 10:00 a.m to 18:00 p.m and indirectly by analyzing brief (up to 600 frames/sec) video sequences recorded with Canon

LEGRIA HF R506 video camera. Recordings were taken during the hottest part of the day (between 10:00 a.m and 18:00 p.m) when the wasps were most active. A total of 600 h of video footage was recorded (279 h in 2016 and 321 h in 2017) and all 121 colonies were observed for the same duration (8 h). Subsequently, two operators independently screened the video recordings using a slow motion system (VLC software v2.2.0) and the agonistic behaviors observed were used to establish an ethogram as described below. The ethogram was supplemented with further “attack” and “defense” behaviors not observed by us but reported in the literature for similar species, or in these two species hornet *V. orientalis* and *V. crabro* facing different antagonists. This approach allowed us to evaluate the repertoire of agonistic behavior between Iranian honeybee *A. mellifera meda* against two species hornet *V. orientalis* and *V. crabro* in a wider context. The frequency (number of events per unit of time) was reported for all the recorded attack and defense behaviors. Attacks at ground level (only on individuals still alive and close to the hive) were monitored in 2016 on the same colonies, concurrently with some of the observations at the nest entrance. These observations were conducted by sight, without using the video camera, for a total of 8h. Two operators simultaneously observed the ground surface under 121 hives from site (A=50) and site (B=71), in two sessions per day, each lasting 15 minute. The frequency (number of events per unit of time) was reported for all the observed attack and defense behaviors.

2.3 Colony observations

Each colony at sites A and B was observed for three different days, 15 min per hour (from 10:00 am to 18:00 pm). The number of hornets visits and number of bee seized, foragers and bees at the entrance (including the presence/absence of particular “bee-carpet behavior” formed by tight groups of workers on the Iranian Langstroth beehive platform or on the vertical walls near the entrance) were estimated for each colony through direct observations and 15 min long video-recording sessions. The environmental temperature was continuously monitored with an electronic thermometer. For 50 beehives (apiary A), we also calculated approximate colony “strength”, i.e. the size and health of the colonies (number of wood frames covered by bee, number of wood frames with immature brood and number of wood frames with food reserves). Finally, we counted the number of dead hornets *V. orientalis* and *V. crabro* (European hornet) found in front of each beehive in two apiaries in Savojbolagh district, sites (A) and (B) in the Alborz province of Iran.

2.4 Bee balling formation and balling temperature

In an experiment conducted on the 50 colonies in site (A), a dead hornet *V. orientalis*, tied to the tip of a 50 cm long thin stick, was introduced 5 cm in front of the beehive entrance. After 10 sec from the onset of bee ball formation, we counted the number of workers involved by putting the bee ball into a nylon bag. For the “temperature balling experiments” we used 71 colonies from site (B). Dead hornet (*V. crabro*) European hornet, (captured directly in the field and rapidly killed at high temperature) were tethered with a fine fishing-line to the tip of a 5 mm diameter, 35 mm long electronic sensor hanging from a 50 cm thin long stick and connected to a thermometer by means of an electric wire. The hornets were suspended close to the beehive entrance (about 5 cm away) and were soon covered with bees. We recorded the temperature

variation inside the hive using two 2 mm micro-probes connected to a highly accurate ($\pm 0.1^\circ\text{C}$) digital thermometer (YCT RS-232 thermometer) [52]. The first probe was attached to the hornet's thorax and the second about 15 cm from the hornet. The rise in temperature in the core of the bee ball, whenever it formed after presentation, as well as environmental temperature, was checked every 30 sec for 15 min. In all experiments the dead hornets were replaced after every three presentations.

2.5 Statistical analysis

The Spearman test was used to check for any correlations between the number of foragers, the presence or absence of "bee-carpets", the presence of bees at the beehive entrance and the rate of hornet attacks, and the number of bees captured. The same test was also applied for testing possible correlations among environmental temperature, honeybee ball core temperatures and the number of bees forming the balls.

3. Results and Discussion

3.1 Reaction and tactic for hornet–honeybee colony

The hornet hunting strategy includes various tactics: European hornet *V. crabro* workers can prey on honeybees (and other insects) whilst engaged in foraging activities, or they hovered near the hive entrance in a search for recently dead, tired and debilitated workers. Rarely, hornets approach the hive entrances to catch freshly landed foragers, but instances of hornets taking bees from groups of individuals "bee-carpets" which sometimes form near the entrance were not observed. More often, hornets catch individual honeybees in flight, hovering in front of the hive entrance and waiting for them to arrive. After catching a bee, the hornet takes it to a perch for processing before taking it back to its nest. The perch may be a tree branch and, in some cases, it is repeatedly used by the same hornet. The predator grasps the bee with its forelegs and first bites the head, killing it instantly. The processing of the corpse continues with the elimination of the head, legs and wings (Fig. 2).



Fig 2: A European *Vespa crabro* hornet is processing a freshly caught bee on the tree branch.

Many studies Rothenbuhler [59]; Stort [64]; Collins [20]; Collins *et al.* [21]; Guzman-Novoa and Page [30], have demonstrated the importance of genetics in honeybee aggressiveness and defensiveness, with some strains being more aggressive than others Ruttner [58] and Breed *et al.* [11]. Direct attacks on hornets by honeybees are also ineffective because of *V. crabro* highly effective predatory behaviors. In some colonies, when attacked, the numbers of guards at the hive entrance increases rapidly to attack, engulf, and kill invading hornets. In other colonies, guards avoid conflicts with hornets by retreating gradually and by forming a defensive line of honeybees at the hive entrance. Sometimes, before departing for its nest, the hornet may cut off the bee's gastrum, after

partially chewing it to extract the honey bag. When a hornet approaches in flight, a group of bees on the platform in front of a hive survey them and perform a kind of "hola" wave by raising their bodies when the hornet hovers near them. This behavior has been noted and described before in *A. mellifera* races with the term "body shaking" and in other species such as *A. cerana*, *A. nuluensis*, *A. dorsata* and *A. florea*. Breed *et al.* [12], summarized these observations as "body shaking", the behavior when "bees massed at the nest entrance or curtaining exposed comb synchronously raise their abdomens, creating a ripple effect that impedes the hornet from landing". The bees which most actively keep the hornet under surveillance are always in contact with other bees in the group. If the hornet gets closer, the bees try to catch it by waving their anterior legs and keeping their mandibles open. They may hold their mandibles open to disperse the mandibular pheromone heptanone, involved in defense functions and in the recruitment of other bees Vallet *et al.* [71]. If some of the defenders succeed in seizing the predator, their rearward mates hold on to them tightly "clinging" behavior; thus chains of bees are formed that can knock the hornet down and subsequently cover it in a group "balling" behavior. Thus, perfect coordination among the defenders, their spatial proximity and the formation of body chains are all necessary to deal with the predator; "ball formation" certainly implies communicative devices to increase the number of bees involved in the defense (Fig. 5C). In fact, if only a few bees are engaged in blocking a hornet, the latter can easily get rid of them and fly away to start hunting again in just a few minutes.

3.2 Defensive reaction honey bee against attack hornets

In response to *V. orientalis* and *V. crabro* the presence around the hive, colonies exhibited three different behavioral patterns. (a) In 50% of observed colonies, a large number of honeybees gathered on the flight board and on the vertical walls near the entrance to form a cluster or a bee-carpet (Fig. 3). (b) In 32% of observed colonies, honeybees on the flight board exhibited a bee-carpet and a coordinated behavior, with individuals clinging together in groups and following the hornet's movements by turning their body in its direction. We did not observe any shimmering in front of the hive entrance, but we did observe frequent hissing. The level of coordination between honeybees varied among colonies and apiaries. (c) The remaining colonies (42%) did not exhibit any coordinated behavior; instead, the honeybees dispersed on the flight board and on the front wall of the hive. During our observations under natural conditions, honeybees rarely abandoned the bee-carpet formation to attack the hornet (Fig. 4).



Fig 3: Illustration is shown a large number of honeybees gathered on the flight board and on the vertical walls near the entrance to form a cluster or a bee-carpet. (Photos by Shakib Vaziritabar, University of Varamin-Pishva in Iran, 2017)



Fig 4: A honeybee colony under attack hornet predation pressure and defense reaction colony against skirmish in front of the hive entrance in the apiary.

3.3 Division of labor during colony defense responses

Honeybee colonies are organized into castes according to a temporal polyethism, with individuals of different ages having different roles in sustaining the community [73]. Two populations of bees that perform nest defense have been described: guards and soldiers or stingers. Here, we will use these denominations for simplicity; however, the most striking feature of these populations is that they are not well defined. In contrast to other eusocial species (e.g. some ants and termites), in which defensive individuals can be highly specialized, guard and soldier bees are not morphologically different from other bees. Furthermore, nest defense is a very transient behavior of honeybees and strongly overlaps with other tasks, particularly foraging; hence, the identity of the defensive bees is constantly changing. Guarding is typically performed by bees during the transition period from inside duties to foraging. Guards can vary greatly in age but are usually 2 to 3 weeks old, and they consistently become foragers after or between guarding bouts. Guards are commonly seen sitting at the hive entrance in a characteristic stance, their forelegs off the ground and their antennae pointing forward (Fig. 5A), or when very excited, with their mandibles open and their wings held away from their body, ready to fly towards any intruder (Fig. 5B). The main roles of guards (described in more detail below) are to check whether incoming bees are their nestmates, and to alert the colony to the presence of a predator. The number of bees allocated to guarding is fairly small, only 10 to 15% of workers become guards Moore *et al.* [44] and usually they guard for no more than a day. However, this number increased after a disturbance or when more intruders are trying to enter the hive [12, 17]. Colonies displaying a stronger overall defensive response tend to allocate more workers to guarding, and these guards remain active for a longer period [15, 29]. The number of guards at the hive entrance correlated with the defensive response of a colony to a disturbance; however, only a small fraction of guards actually participates in the stinging responded. Thus, the main function of guards may be the detection and signaling of threats. There is some evidence that another population of bees referred to as ‘soldier bees’ is responsible for harassing any intruders, but this remains a subject of debate. The degree of wear of soldiers’ wings is significantly lower than that of foragers of the same age, so it has been suggested that these bees spend more time inside the hive, where they can be quickly mobilized to the entrance [12, 14]. In addition, the propensity to sting is regulated by both genetic factors and age, with older bees being more likely to sting [31]. Indeed, a number of studies have also demonstrated a patrilineal effect, and have mapped quantitative trait loci that are associated with guarding, stinging or both behaviors

[6, 11, 30, 34, 41, 60]. More recently, a transcriptional ‘signature’ of aggression has been identified in the bee brain [5, 18]. In this review, we will not include further detail regarding the genetics of honeybee aggression, as this has been extensively reviewed previously [11, 34]. At first glance, the overall defensiveness of a colony correlated with the individual responded of its members to noxious stimuli Avalos *et al.* [4], but the link between defensiveness at the individual and at the colony level is far from simple. Complex interactions between individuals are also at play, as evidenced by cross-fostering experiments showed that bees from an aggressive genetic background tend to take over guarded when raised in more gentle colonies, and inversely, gentle bees are less likely to guard when placed in aggressive colonies [13]. In parallel, cross-fostered bees seem to adopted the propensity to sting of their host colony to some extent Guzman-Novoa and Page [31] and Paxton *et al.* [55], which suggests that guarded and sting were differentially regulated but both dependent on colony environment. Finally, when the most aggressive bees of a population are removed, the remained ones then take over defensive tasks.

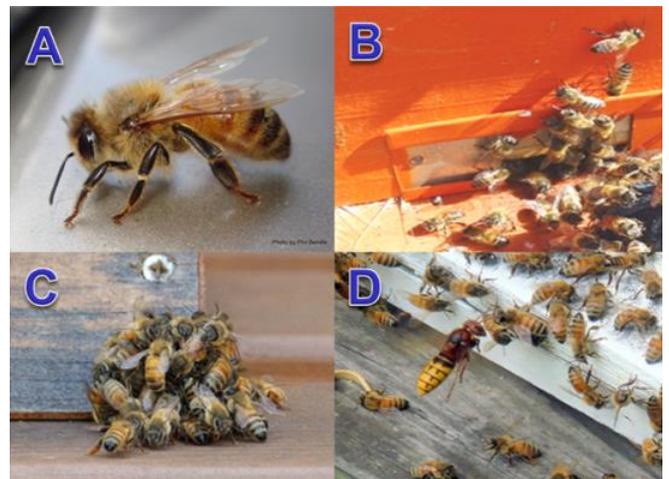


Fig 5: Illustrations of differential defended reaction and tactic of Iranian honeybee *Apis mellifera* meda colonies under attack hornets *V. orientalis* and *V. crabro*. (A) Guard in the characteristic stance, forelegs off the ground and antennae pointing forward. (B) Alerted bee ready to fly off toward the intruder. (C) Honey bees engulfed a hornet in a ‘hot bee ball’. (D) A honeybee colony under attacked *V. crabro* predation pressure and European hornet (*V. crabro*) is visible in the background Iranian langstroth hive.

3.4 Division reaction and tactic Iranian honeybee against attack hornets

Unfortunately, No studies exist regarded the defense behavior of Iranian honeybee *Apis mellifera* meda against attack hornets *V. orientalis* and *V. crabro* in Iran and no previous studies have described behavior of area guards in Iran. The defensive behavior of the Iranian honeybee *Apis mellifera* meda reveals some similarities and basic differences compared to other *Apis mellifera* subspecies facing different *Vespa* species. For example, in a recent study, Baracchi *et al.* [10], described in details the behavioral interactions between *Apis mellifera* ligustica preyed by *Vespa crabro*. Like in Cyprus, the Italian honeybees demonstrate massive defensive behavior expressed through coordinated “body shaking” from the “bee-carpet” formed in front of colonies’ entrances. In addition, they perform balling behavior in many cases. In Alborz apiaries in Iran, balling behavior was present in attacker colonies but it was absent in retreaters. The “bee-carpet” was present before the approach of predators in

colonies, independently from the anti-predator tactic each colony expressed, as can be seen by the initial numbers of guards at the flight boards, but the behavior changed upon the hornet's approach. Attackers recruit more bees and "attack" the hornets while retreaters gradually deform the "bee carpet and retreat behind the propolis walls". Probably, the differentiations on defensive behavior of *A. m. meda* (Iranian race honey bee) have a result of co-evolution and adaptation to the different predation tactics expressed by *V. orientalis* compared to *V. crabro*. *Vespa. crabro* never attacks and catches individual honeybees from the "bee carpet" formed in front of the entrance [9]. This is the rule in *V. orientalis*; they attack directly the honeybees forming the "bee carpet", trying to catch an individual and escape, avoiding further conflict with the defenders. Furthermore, *V. orientalis* rarely attacks the few foragers departing or returning from foraging activities (mainly at the colonies expressing the "attacker" behavior) while *V. crabro* preys on honeybees engaged in foraging activities. A few workers of the Japanese giant hornet *Vespa mandarinia* can exterminated a large honeybee colony within a single day, and later feed on the pupae and larvae [46]. Because of the hornets' hard cuticles, it is nearly impossible for honeybees to sting them. Thus, the bees' defensive behavior during such attacks first involves forming large aggregations at the hive entrance. The bees cling to each other to form a 'carpet' and try to catch the hornet with their front legs and mandibles. If successful, they will then quickly trap the hornet within a dense ball of bees [10]. Interestingly, this behavior is widespread throughout the *Apis* genus but has evolved to fit the particular interactions of each honeybee species/subspecies with the corresponding local species of hornet. *Apis mellifera meda* honeybees, which originate from Asia, where there are six species of hornets, are particularly efficient in recruiting over 30 workers to form a 'living ball' inside which the hornet is trapped and killed by the high core temperature of approximately 45°C. Bees achieve this increase in temperature by contracting their thoracic muscles. The temperature in the center of the ball is above the thermal limit of the hornet, yet it is harmless for the bees themselves, which have a thermal limit of approximately 50°C [39, 49]. Iranian honey bee race *Apis mellifera meda* also use this strategy to confront *V. crabro*, a mild predator that occurs in the native range of this subspecies, although only 15 to 20 workers are involved (Fig. 5C), and they raise the ball temperature to 44°C only [10, 38]. Another subspecies, *Apis mellifera cypria*, is confronted by *V. orientalis*, which has a thermal limit similar to that of honeybees. Consequently, these bees block the hornet's respiration by inhibiting the pumping movements of its abdomen in addition to increasing the temperature, thus asphyxiating it [53]. Alternatively, some colonies of this subspecies retreat behind propolis walls with narrow, easy to guard openings at the hive entrance and never try to engulf the hornet [51]. The reason for the co-existence of these different strategies remains unknown. Honeybees have been reported to produce piping sounds or 'hisses' when hornets are around, also described as 'shimmering' [10, 52]. Hissing seems to be an innate response to noxious stimuli, as this behavior is also produced in response to electric shocks [72]. Whether these sounds are used as an alarm signal to the colony, as a threat to hornets (which are known to use high-frequency sounds for communication) or are just distress

sounds remains to be determined.

3.5 Visiting frequency of each *Vespa* species in the apiary

The hornets start to visit the apiaries early in the morning at about 10:00 am, going on until about 18:00 pm and reaching maximum activity during the warmest hours of the day (13:00 p.m. to 15:00 p.m.). From 10:00 am to 18:00 pm, the number of hornet visits to each colony during the 15 min observation periods was 32.4 ± 24.5 (n = 50) in apiary (A) and 9.4 ± 6.30 (n = 71) in apiary (B), while the number of bees caught was 2.6 ± 1.42 and 0.8 ± 0.14 respectively, (Table 1). We can estimate that on average the beehives in the two apiaries suffer a loss of up to 84 ± 15.6 and 18.4 ± 7.9 bees in a single day respectively, and that the hardest-hit beehives undergo a loss of up to 80 and 41 bees, respectively.

Table 1: Mean and standard deviation of number of hornet visits, number of caught bees, number of guards and number of foragers counted in the beehives in Savojbolagh apiaries (A and B).

Data item	Savojbolagh apiaries in Alborz province	
	Site A (n=50)	Site B (n=71)
Number of hornets visits	32.4 ± 24.5	9.4 ± 6.30
Number of caught bees	2.6 ± 1.42	0.8 ± 0.14
Number of guards in colony	645.36 ± 218.88	218.33 ± 38
Number of foragers	660.58 ± 218.23	341.6 ± 104.62

Note: Number of foragers counted in the beehives in Savojbolagh apiaries (site A and B) in Alborz province. (Source: Field Survey, 2016).

At the apiary sites, 2 wasp and bee species were collected, including 2 species of vespidae and one species of Apidae (Table 2). In total, 2 species were found in the apiaries and two wasp attacked beehives and predated on honeybees (Fig. 6). We found five hundreds dead honey bees piled up; they must have been attacked by a group of *V. crabro*. As shown in (Fig. 5D) provided that each individual foraged for honeybees one at a time. In the presented study, however, many workers attacked the apiary since July. In June, there was a rapid increase in the number of *V. crabro* visiting the apiary; this number reached a peak in August, which lasted into September. Consequently, *V. crabro* most frequent hornet and next *V. orientalis* was the second most frequent *Vespa* species in the Savojbolagh apiaries. Therefore, we considered *V. crabro* being a new pest of honeybees; the impact of this species on Savojbolagh apiaries is considerable in Iran. *Vespa. crabro* occurred most often in the apiary and caused serious damaged (Fig. 5). We only observed seven instances in which a hornet accidentally came into contact with the bee-carpet; of these seven instances, guarded bees only once engulfed and transported the hornet inside the hive. In those hives without any coordinated colony defended, we observed that individual bees were sometimes isolated on the flight board and on the wall of the hive. In such situations, the hornet shown particular interest in the lone bees (Fig. 5D). When the colony activity was very low and no bee-carpet formed at the hive entrance, hornets entered the hive to steal pollen and honey stores, as well as larvae and pupae. The honey was consumed on site and exchanged with other hornets by trophallaxis. Trophallaxis is the only cooperative behavior that we observed among hornets in front of the hive

Table 2: Foraging behavior of wasps and a bumble bee (*B. terrestris*) in the apiary.

Vespa species	Preying on Honeybees		Foraging Behavior	Skirmish & Steal Honey	
	Alive	Dead		Abandoned comb	Inside hive
Vespinae					
<i>Vespa crabro</i>	○	-	L	○	-
<i>Vespa orientalis</i>	○	○	L&H	○	○
Apidae					
<i>Bombus terrestris</i>	-	-	-	○	-

Note: L, landing; H, hawking.

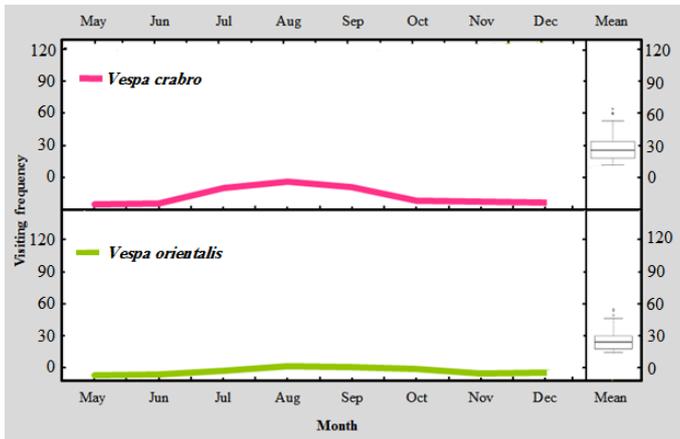


Fig 6: Monthly visiting frequency of each *Vespa* species over 8 h in the beehives in Savojbolagh apiaries (A) and (B) from May to December 2017.

In most cases these actions were limited to flight clashes but on two occasions we observed a fierce battle which, in one case, ended with the death of one of the wasps. This fact suggests that some of the hornet corpses found near the hive entrances could also be due to competition between workers from different *V. crabro* colonies. The number of hornet visits to the beehives was strongly correlated with the number of foragers (Spearman correlation test, $n = 121$, $r = 0.95$, $P < 0.001$), the number of bees caught (Spearman correlation test, $n = 121$, $r = 0.77$, $P < 0.001$) and the size of the “bee-carpet” (Spearman correlation test, $n = 121$, $r = 0.55$, $P < 0.001$). Moreover, “bee-carpet” size near the hive entrance was also correlated to the number of foragers (Spearman correlation test, $n = 121$, $r = 0.98$, $P < 0.001$). Interestingly, the number of foragers was significantly correlated with colony “strength” (see Materials and Methods) ($n = 121$, $r = 0.86$, $P < 0.001$), furnishing a good external estimate of colony quality. We directly observed seven cases in each apiary in which assaulting hornets *V. orientalis* emballed and killed by the bees. The balls dissolved after a minimum of roughly 30 min, leaving the wasps dead or dying. As shown in (Fig. 6), hornet visits varied considerably among the hives: many beehives received no visits at all, while others received a high rate of hornet visits, in spite of the fact that all the hives were next to each other in a row. As the hornets were not marked for our observations, we cannot say whether the hornets preferred any particular colony in general (i.e. a lot of different hornets independently plundered the same beehives), or not (i.e. a few hornets repeatedly plundered the same colony they recognized).

3.6 Bee ball formation and honey bee roast

Iranian honey bees cannot kill the hornet by stinging and also some observations made on hornets trapped in balls for short

periods of time (10-15 minute) have also showed that the invader remained motionless for a few minutes before recovering. When the honey bees detect these pheromones, one hundred twenty one or so honey bees will gather near the entrance of the nest, apparently to draw the hornet further into the hive. As the hornet enters the nest, a large mob of about hundred honeybees surround the hornet, completely covering it and preventing it from moving, and begin quickly vibrating their flight muscles. This has the effect of raising the temperature of the honeybee mass to 47°C. Though the honeybees can tolerate such a temperature, it is fatal to the intruder, which can handle a maximum temperature of about 45°C, and is effectively baked to death by the large mass of vibrating bees. The wasps died at 45.7°C, but the Asian honeybees survived temperatures up to 50.7°C and the European bees survived up to 51.8°C. Iranian honey bees (*Apis mellifera meda*), have been showed to kill *V. orientalis* by ‘thermo-balling’, in which they surround a hornet to from within the temperature increases to a lethal level. When killed hornets were presented at the beehive entrance, guards and other honeybees engulfed them in balls of 47.5 ± 8.4 workers within 10 min ($n = 121$, range 6–58), but in another experiment 184 bees were counted in the ball. This accounts for the great variability in response to a possible threat from different colonies. A similar variability was recorded in the temperatures reached in bee balls formed by different colonies in the experiment sites (A) and (B) with dead hornets mounted on a thermal sensor (Fig. 7). On average, at the end of 15 min the maximum temperature recorded at the core of the bee balls was 40.5 ± 7.8 °C ($n = 121$). Nearly 46% of colonies could reach temperatures of over 43.7 °C, while the other 57.5% could not. The highest temperature measured in the honeybee ball core was 45.7 °C in a single colony. The environmental temperature during the experiment averaged 27.35 ± 5.2 °C, while the difference between the environment and bee balls temperatures averaged 27.21 ± 10.6 (range = 1–40). The number of worker bees forming the ball around the hornet was strongly correlated to the maximum temperature reached by each colony (Spearman correlation test, $n = 50$, $r = 0.86$, $P < 0.001$). A strong correlation was also found between the maximum temperature each colony reached and the environmental temperature (Spearman correlation test, $n = 71$, $r = 0.87$, $P < 0.001$). The temperature inside defensive honeybee balls increased rapidly. After the first 8–10 min, the temperature remained stable for about 15 min and then decreased slightly, but it never returned to its initial value.

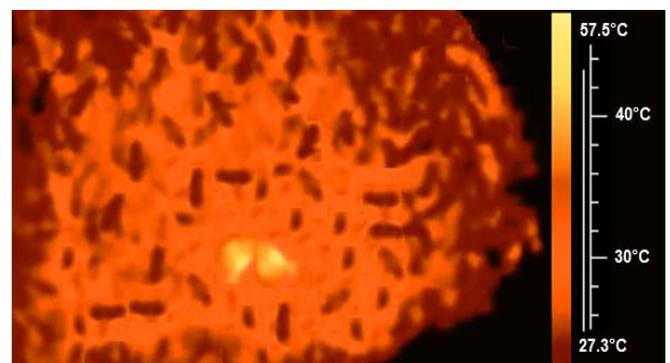


Fig 7: Iranian honey bees (*A. m. meda*), have been showed to kill hornets by ‘thermo-balling’, in which they surrounded a hornet to from within the temperature increased to a lethal level and unusual thermal defended by honey bees against mass attack by *V. orientalis* hornet. Hornets asphyxiated by *A. m. meda*. (Photograph by Shakib Vaziritabar, 2017).

3.7 Determination of *V. crabro* lethal thermal limit

The lethal thermal limit of *V. crabro* determined with our measurements was approximately 42.9 ± 0.9 °C ($n = 7$); different rates of temperature increased did not influenced the lethal thermal limit (Spearman correlation test, $n = 7$, $r = 0.009$, $P = 0.87$). The lethal temperature refers to the complete immobilized of the wasps, yet above 43.7 °C their vital capacities already seemed compromised. We demonstrated that *A. mellifera* meda in Savojbolagh apiary (site B) in Alborz province produced a maximum temperature that could not sufficient to kill the *V. crabro* hornet. The absence of movements reduces convection and contributes to an efficient use of the heat. The bees surrounding the core are an active insulating layer in so far as their heated thoraces reduce the thermal gradient. However, while incubator experiments indicate that the temperature could be lethal; other possible reasons in natural conditions cannot be excluded, like the production of CO₂ or limits to the function of the hornet's respiratory system or the beestings and the toxic action of the venom. The present study obviated that the gradual increased in the numbers of attacking hornets against honeybee colonies with the progressive season during the two monitoring years was coincided with the progressive recruitment of adult hornets for collected extra food as a response to demands of their larvae within nests. The size of hornet nest increased as the season progresses, so, it contained a great numbers of hungry larvae which stimulate the foraging activity of adult hornets. These explanations are in accordance with findings of many researchers [2, 67, 75]. They recorded gradual increase in the foraging of oriental hornet from early summer until registered the highest population during July to October. Also Sharkawy [67] and Ishay [35], reported that the highest number of hornet brood within the nest was found during the month of October. Another defensive strategy is surrounding hornets by a group of workers in a tight ball and used their thermoregulatory capabilities to raise the temperature inside the ball to a lethal point for the hornet [1, 49]. The colony strength (worker population) may also play a satisfactory role in colony defense when conflicted with hornet attackers. The total predation of honeybee workers from colonies with higher population was significantly less than that recorded for colonies with lower population. However, there is no consensus in the literature; some studies found a negative correlation Giray *et al.* [32] while other found a positive correlation Wray *et al.* [76] or no relation at all Page *et al.* [56]. The present study showed that the demonstrated a strong correlation between the number of hornet visits to a particular beehive and the number of incoming and outgoing foraging bees and we did not observed a hornet plundering a bee from a "bee-carpet" also suggests that this aggregation is a defensive strategy against these predators, although clusters of bees in the front of the hive may occur for other reasons, for example induced by particular climatic conditions. "Bee-carpets" are in fact larger in beehives with higher forager flow (where hornet predation is also high) but carpet size is not related to any other parameter, including colony size and environmental temperatures. Dead hornets found that the large numbers under some beehive platforms and direct observations of events demonstrate that *A. m. meda* is able to counteract *V. crabro* predatory pressure with a defense strategy. Part of this strategy was comprised of knocking down and killing the flying hornets through coordinated honeybee group activity. As already mentioned, "balling" behavior is described in *A. mellifera* against *V. velutina* Ken *et al.* [37] and in the subspecies *A. m. cypria* against *V.*

orientalis Papachristoforou *et al.* [53]. However, while the latter study shown that how the particular balling behavior of *A. mellifera* meda is adapted to the biological characteristics of its predator (very high lethal thermal threshold compared with that of *V. crabro*), the first study stresses the poorly adapted response of *A. mellifera* towards a non-co-evolved predator like *V. crabro*. Balling behavior in *A. m. meda* seemed quite adequate to counteract *V. orientalis* predatory behavior, providing a sign of long co-adaptation in a temperate environment, while it seemed totally inadequate against other predators such as *V. crabro* [24]. Balling bees can kill *V. orientalis* hornet worker in less than 20 min, although *A. m. meda* in (site B) in Alborz province produced a maximum temperature that could not sufficient to kill the *V. crabro* hornet. However reaching temperatures that in our experiments approached the lethal thermal limits of the wasp. Eventually, *A. m. meda*, have been showed able to kill *V. orientalis* by 'thermo-balling', in which they surround a hornet to from within the temperature increases to a lethal level but inefficient and unorganized defense against European hornet *V. crabro*. Iranian honey bee (*A. m. meda*) showed a distinct balling reaction against workers of the predatory various races hornets *V. orientalis* and *V. crabro* killing the hornets by heat and suffocation generated inside the ball. Considering that the duration of bee balls is far longer than the experimental times, we maintain that overheating is probably sufficient to cause the death of the predator.

4. Conclusion

The present study concluded that the colonies showed a lower defensive response when bees performing guarded behavior were removed from the colony. The variation in the defensive level of the colonies with or without guards suggests that guards play an active role in the defensive response of a honey bee colony. In *A. m. mellifera*, defense behavior is complex could be genetically determined and shows potential high level of heritability. Especially, one might consider that selection on specific behavior such as the balling behavior could help enhancing the defensiveness of the honeybee colonies. The bees surrounding the core are an active insulating layer in so far as their heated thoraces reduce the thermal gradient. This way they actively conserve the heat of the core bees because it is only the heat of the clinging bees that kills. The insulating layers of honeybee winter clusters, by contrast, reduce endothermy to a minimum to prevent heat loss. Lethal temperatures of different wasp species attacked via thermal balling are in the range of 44–46 °C. Our attacked wasps did not reach this level but we suggested that they were warmer during times of maximal heating when the balls were too dense to identify them properly in the thermograms. Finally, the present study provides insight into the mechanisms of attack and defense deployed by *A. m. meda* against two hornets *V. orientalis* and *V. crabro* both in terms of predator prey co-evolution and in terms of potential defense strategies that can be used by native bees against alien species such as *V. orientalis* and *V. crabro*. Although these results are preliminary and should be carefully considered, thus further experiments are necessary to confirm this hypothesis.

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